Draft Discussion Notes: GRN 162: Complicating the Record

1. Letter dated 2/4/05 from Covington and Burling and resent under Ruth Miller's signature on 5/19/05.

   A legally based argument based on the premise that filing an IND would constitute a legal basis for restricting use in foods as a GRAS substances as well as restricting use as a dietary ingredient in dietary supplements.

2. Letter dated 5/20/05 from Agennix, Inc.CEO Richard Barsky. Purported "scientific" argument that safety has not been shown based on use of comments to APHIS docket on EA for the rice used to produce lactoferrin. Attachments: Comments to APHIS docket on "lactoferrin rice" from Friends of the Earth (Bill Freese), Center for Food Safety (Doug Gurion-Sherman and Peter Jenkins), and Consumers Union (Michael Hansen)

   "These public documents were submitted by respected consumer advocacy groups in response to Ventria's requests for approval to grow GMO Pharma rice containing recombinant human lactoferrin. The documents point out that recombinant human lactoferrin has not been shown to be safe for general human consumption, and in fact, depending on the full recombinant sequence (which Ventria has never determined or disclosed) and the specific glycosylation, it could be hazardous. There clearly is not a consensus within the scientific community that recombinant human lactoferrin is safe for its intended use and thus it fails to meet the requirements for being considered GRAS."

   In GRN 159 Ventria presents evidence of the sequence and discusses glycosylation and why glycosylation should not except in special circumstances raise safety questions.

3. Letter dated June 1, 2005 from Agennix, Inc., CEO Richard Barsky. "Safety risks of "recombinant human lactoferrin" and concerns about allergenicity of rice proteins."

   In GRN 159 Ventria discusses antilactoferrin antibodies in autoimmune diseases and that they are considered an epiphenomenon without a clinical consequence. Ventria also plans to have the LF source labeled for rice, because it acknowledges that some rice proteins are present in the final preparation.

Agennix Incorporated

OU

RECD JUN 3 2005

June 1, 2005

Laura M. Tarantino, Ph.D. (HFS-200)
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Agennix

Re: **Ventria GRAS Notice No. 162: Use of Recombinant Human Lactoferrin In Food and Dietary Supplements**

Dear Drs. Tarantino, Schneeman, and Martin,

With respect to the petition for GRAS listing of recombinant human lactoferrin (rhLF) by Ventria BioScience (GRAS Notice #162), we respectfully submit the following additional public document for consideration:

"Human lysozyme and lactoferrin therapeutic proteins also have been implicated in pathological conditions": Comments submitted to USDA/APHIS Docket Nos. 05-006-1 and 05-007-1"

Professor Joe Cummins, The University of Western Ontario – April 2, 2004

Eight Greenway Plaza Suite 910 Houston Texas 77046 Telephone 713/552-1091 Facsimile 73/552-0795

This public document was submitted by a respected scientist in response to Ventria's requests for approval to grow GMO Pharma rice containing recombinant human lactoferrin. The document points out that recombinant human lactoferrin has not been shown to be safe for general human consumption, and in fact could be hazardous.

In addition to the potential safety risks of recombinant human lactoferrin, we also wish to point out that Ventria's proposed production in rice entails additional health risks. The frequency of IgE -mediated rice allergy can be as high as 10% in atopic subjects and lead to serious complications including asthma, diarrhea, vomiting, atopic dermatitis and exercise-induced anaphylaxis [Internet Symposium on Food Allergens 1(4):147-60 (1999)]. Making Ventria's recombinant human lactoferrin broadly available through a GRAS listing would greatly increase the probability that individuals allergic to rice antigens would inadvertently be exposed to dangerous allergens.

We believe the information submitted currently, together with our previous submissions, clearly demonstrates that **Ventria's petition for GRAS listing of recombinant human lactoferrin should be DENIED.**

Please do not hesitate to contact us at the address or phone number below if we can provide any additional information that would be helpful.

Sincerely,

Rick Barsky
Chief Executive Officer

Attachment

Eight Greenway Plaza Suite 910 Houston Texas 77046 Telephone 713/552-1091 Facsimile 73/552-0795

April 2, 2004

Human lysozyme and lactoferrin therapeutic proteins also have been implicated in pathological condition

By Prof. Joe Cummins
e-mail; jcummins@uwo.ca

Recently crop plants modified with the therapeutic proteins lysozyme and/or lactoferrin have been tested in the field. Rice modified with lysozyme and lactoferrin has been put forward for commercial production(1). Lysozyme protects us from the danger of bacterial infection. It is an enzyme that attacks the protective cell walls of bacteria. Bacteria build a tough skin of carbohydrate chains, interlocked by short peptide strands, that braces their delicate membrane against the cell's high osmotic pressure. Lysozyme breaks these carbohydrate chains, destroying the structural integrity of the cell wall. The bacteria burst under their own internal osmotic pressure.

Lysozyme is present in bacteria and animals alike, birds egg lysozyme is a powerful food allergen but human lysozyme is less likely to cause allergy. Lactoferrin is a protein that participates in regulation of immune functions and controls pathogens by binding iron required for bacterial growth.Both lysozyme and lactoferrin are present in mother's milk and both are used to treat infections.

In spite of their value in controlling infection there are pathological conditions associated with the proteins are genetic variants of the proteins. These pathological associations seem to have been overlooked by the promoters of the biopharmaceutical applications of proteins. However, the pathological side effects should have been considered because food crops may become polluted with the genes for the proteins and the proteins. The references below may provide useful information for those questioning the production of the proteins in food crops.

Lysozyme provides effective contol of many infections but the protein may as well, contribute to the pathology of pulmonary emphysema by binding to elastic fibers which undergo breakdown in the disease (2). Hereditary systemic amyloidosis is caused by mutant forms of cell proteins deposited as amyloid fibrils, the mutant cell proteins include lysozyme or apolipoprotein or fibrinogen (3). The lysozyme related disease was provoked by a single mutation of tryptophane to arginine. The disease is inherited as dominant gene and frequently leads to death at mid-life (4). A lactoferin mutant has been implicated as a cause of amyloidosis accompanied by trichiasis (a common vision threatening condition of the eylid). The lactoferrin mutant resulted from a single change from glutamic to aspartic acid near the end of the protein molecule (5). Lactoferrin has also been found to be implicated in forms of autoimmune diseases including systemic lupus erythematosis and rheumatoid arthritis (6) or in rheumatoid arthritis associated with vaculitis (inflammation of blood vessels) (7).

Production of human lysozyme and lactiferrin in food crops such as rice, barley or maize has been promoted as if the products purely beneficial without harmful side-effects. However, there is evidence that moth normal and mutant forms of the proteins are associated with serious detrimental human diseases. In particular the synthetic forms of the human genes used in crop modification are modified both in codons and frequently amino acids to enhance production of the proteins in plants and these alterations are seldom thoroughly tested. Mutations of the human genes in the plants are not normally identified unless they eliminate production of the protein.

There seems to be a culture that puts optimism ahead of experiment and distains labeling of products, crop production or field tests so that deleterious side effects of the crops or their products cannot be identified.

Why is the evidence of harmful side effects and dangerous mutant forms [not] presented at the safety evaluation of field tests and production sites? The answer seems to be that both proponents and regulators do not wish to alarm the public. Indeed, if and when the matter is brought up regulators and proponents will unleash teams of vicious lawyers whose job is to shift the burden of proof to those who mention harmful or dangerous side effects .

Nevertheless, if and when the dangerous tests or crop productions are undertaken people who are effected should begin to notice amyloidosis or autoimmune diseases including lupus and arthritis. The main geographical areas of concern are California where biopharmaceutical rice is being "tested" on a large scale and in Washington state where large plots of biopharmaceutical barley is being "tested".

References

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2. CANTOR,J.,SHTEYNGART,B., CERRETA,J. AND TURINO,G. "The Effect of Lysozyme on Elastase-Mediated Injury" 2002 Exp Biol Med 227:10813

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4. VALLELX,S., DRUNAT,S., PHILIT,J., ADOUE,D., PIETTE,J., DROZ,D., MACGREGOR,B., CANET,D., DELPECH,M. and GRATEAU,G. "Hereditary renal amyloidosis caused by a new variant lysozyme W64R in a French family" 2002 Kidney International 61,907-12

5. Ando,Y. "Analyses of pathogenesis and therapeutic approaches for hereditary amyloidosis" 2003 Rinsho Byori 51,530-5

6. Nassberger L, Hultquist R and Sturfelt G. "Occurrence of anti-lactoferrin antibodies in patients with systemic lupus erythematosus, hydralazine-induced lupus, and rheumatoid arthritis." 1994 Scand J Rheumatol. 23,206-10

7. Coremans I, Hagen E, Daha M, van der Woude F, van der Voort E,

Kleijburgvan der Keur C, Breedveld F. "Antilactoferrin antibodies in patients with rheumatoid arthritis are associated with vasculitis." 1992 Arthritis Rheum. 35,1466-75

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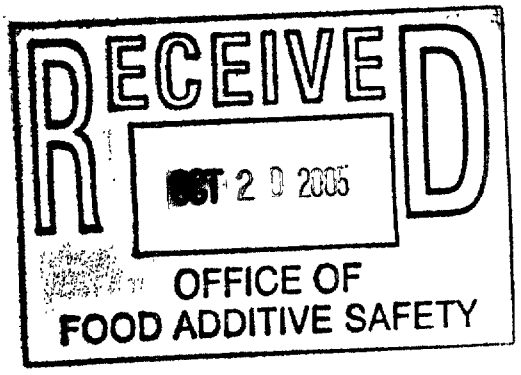
October 19, 2005



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Re: Pharming Group N. V.
Comments in response to comments filed in opposition to CFSAN "approval" of GRAS Notification number 162

Dear Drs. Tarantino and Mattia:

On December 22, 2004 Ventria BioScience filed a GRAS Notification with CFSAN pertinent to use of its human lactoferrin product in food. CFSAN subsequently entitled such Notification GRN No. 162.

Subsequently, Covington & Burling and one of its clients, i.e., Agennix Incorporated, filed comments pertinent to GRN No. 162 (on February 4 and May 19, 2005 and on May 20 and June 1, 2005 respectively) which – in essence – requests that CFSAN deny Ventria's request for GRAS status of its human lactoferrin product. Since such comments also specifically reference Pharming and its future intent to file a GRAS Notification concerning its human lactoferrin product for use in food **and,** thus, make such comments pertinent also to Pharming's future notice, Pharming has requested that I – as their US regulatory counsel – respond to the filed comments. Thus, what follows are these comments.

First, Pharming has **no** position on whether Ventria's notification should be "approved" or "denied" by CFSAN. Pharming expects CFSAN to review such notification (and all such notifications) consistent with **only** the regulatory requirements pertinent to GRAS filings and to base its final decision **solely** on whether Ventria (or any similarly situated petitioner) has met the legal burden emanating from such requirements.

Second, to the extent that the above-referenced comments argue the obvious – and they, in part, do – that is, that if one seeks to place a product into interstate commerce which product is adequately associated with a claim or claims that legally amount to a claim or claims pertinent to, for example, an infant formula, a health claim, a dietary supplement or a drug, then such entity must comply with all regulatory requirements pertinent to the specific claim or claims in question, Pharming agrees with such statement(s). It's **not** clear from a review of Ventria's GRAS Notification that Ventria is seeking to avoid or ignore such requirements; however, CFSAN can assure that Ventria does not simply by including appropriate language in any final GRAS "approval" letter (if such letter is otherwise to be

issued) similar to the language CFSAN included in its "approval" letters to those seeking GRAS status for their bLF products.

Third, the comments argue that "recombinant human LF is not now, and has not ever been, used as an ingredient in food...". However, to the extent that any hLF product is specifically shown to be identical to or substantially similar to native hLF then such argument is scientifically incorrect since hLF has been safely consumed as a part of food, i.e., mother's milk, for thousands of years – just as bLF has been.

Fourth, the argument that once a substance is associated with an approved IND it cannot be used **in any way** as a component of food is nonsense and **not** supported by any law, including Section 201(ff) (3) of the FD&C Act. As the comments conveniently fail to mention, CFSAN currently has full and express authority to "approve" a substance for food use – even though authorized for investigational use – under Section 201 (ff) (3)(B)(ii) of the FD&C Act.

Fifth, the comment that all safety requirements pertinent to use of a substance as a drug must also be applied to that same substance if used as a component in food is not supported by any current law (and **none** is cited in support of such statement). A substance intended for use as a drug must meet all pertinent regulatory requirements applicable to drugs while the same substance intended for use merely as a component of food must meet the requirements pertinent to food and – to the extent applicable – requirements pertinent to whether a food additive or a GRAS substance.

Sixth, the comments seem to argue that either Ventria or Pharming or both are attempting to commercialize their respective hLF products without first interfacing appropriately with CFSAN. At least with regard to Pharming, such a suggestion is not accurate. Ventria has filed a GRAS Notification pertinent to its product (and had done so prior to the filing of the above-referenced comments) and Pharming intends to file a GRAS Notification pertinent to its.

Seventh, in contradictory fashion, the comments acknowledge that – under current food law – one can have a substance to be used in food either approved by CFSAN as a food additive or determined by CFSAN to be GRAS, **but then** argue that the hLF products must be regulated only via food additive petition and then argue that such products cannot be "approved" at all in any fashion. As CFSAN

knows from decades of applying currently, pertinent, legal requirements to both food additives and GRAS substances, only the first argument is correct. As has been known for decades, if one can show (via the regulatory requirements pertinent to GRAS determinations) either via "experience based on common use in food" or on "scientific procedure" (or on both) that the use(s) of a substance in food is safe under the conditions of its intended use, such use can be determined to be GRAS – if the determination is made by a consensus of qualified experts. (Section 201(s) of the FD&L Act).

Eight, the comments acknowledge that there exists a valid "pertinent exception", i.e., a GRAS determination, to the general food additive rule, but argue that the commenter's review of "the published literature" does not qualitatively or quantitatively support the safety of either Ventria's or Pharming's product. With all due respect to such "review", it is the data and information actually in a GRAS Notification – not otherwise – that legally determines GRASness, and Pharming's GRAS Notification will be filled with as much published, quality information – if not more – than has ever been presented to CFSAN in such a submission. To suggest that there currently exist a paucity of relevant information in the scientific literature is to have missed over 1,000 pertinent published scientific papers.

Ninth, the comments seem to argue that either Ventria's or Pharming's products or both may differ from native hLF and therefore, cannot be shown to be safe. If the information in a GRAS Notification adequately demonstrates safety and general recognition (as these terms have been applied for decades), then the subject of such notification can – indeed should be – deemed GRAS regardless of whether the subject form is exactly like the native form. Of course, to the extent that the subject form is substantially equivalent or identical to the native form will presumably be of interest to all parties concerned – including the qualified experts.

Tenth, the comments indicate that only "some products" (indicating a very small number) derived via recombinant activities have been approved by FDA, and then list only three drug products. Rather than citing irrelevant, approved drug products, perhaps the commenter should have cited the over twenty substances intended to be used in food that have been determined to be GRAS by CFSAN – **all** of which emanated from recombinant technology and **all** of whose safety –

contrary to the commenter's assertion (see footnote 23) – was based solely on "scientific procedures" (not – as asserted – on "long marketing histories").

Eleventh, the comments seem to want to differentiate hLF from bLF probably because the arguments contained in the comments are weakened by the fact that bLF has been determined to be GRAS. And importantly – and regardless of whether bLF and hLF are identical – bLF has been repeatedly determined by CFSAN to be GRAS based solely on scientific procedures (**not** on any experience based on common use in food).

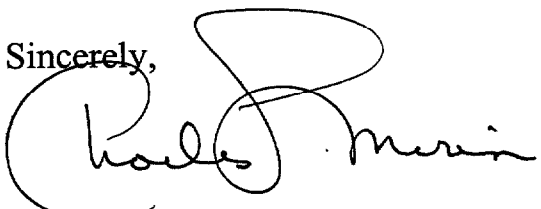
In summary, Pharming has not sought and will not seek to avoid any U.S. regulatory requirements pertinent to lawful use of its hLF product in food in the U.S. Indeed, to that end, it has repeatedly communicated with CFSAN over the years and even met with CFSAN (as long ago as in January of 2001) to make sure that it was pursuing the proper regulatory pathway for use of its product in food. It will continue to do just that.

* * * * *

If after receiving the foregoing information you should have questions or need additional information, please let me know.

Thank you in advance for considering the above-referenced information.

Sincerely,



Charles L. Morin

PR

MEMORANDUM OF MEETING

Date of meeting: April 21, 2004

Time: 12:30 p.m. to 1:30 p.m.

Location: Room 1295, Vermont Ave. Building

Participants:

Ventria Biosciences

Delia Bethell
Scott Deeter

FDA/CFSAN/DBGNR (HFS-255)

Jason Dietz
Michael DiNovi
Feleke Eshete
Rebecca Edelstein
Jeremiah Fasano
Jeanette Glover Glew
Rudolph Harris
Robert Merker
Jeremy Mihalov
Penelope Rice

Subject: Pre-notification meeting: Discussion about scientific aspects of subject of proposed GRAS notice for lactoferrin with an aa sequence identical to that of human lactoferrin.

Dr. Merker began the meeting by informing the representatives of Ventria Biosciences (Ventria) that FDA would be primarily in "listening mode" and would provide Ventria with FDA's response to their quesions after internal deliberation.

Dr. Bethell began by saying that Ventria's product plans are for human proteins made in rice, then extracted from rice grains, and added to food. The subject of this meeting would focus on one such protein (lactoferrin).

Dr. Bethell's presentation focused on the following information:

1. Comparison (similarities and differences) between the native human protein (from human milk) and the rice-derived protein.

a. Differences included glycosylation (i.) rice-derived protein lacks terminal sialic acids found in human proteins, ii) a xylose present in the plant structure usually is absent from the mammalian structure and iii) fucose is linked though a different linkage. These differences result in a slightly smaller molecule.
b. Ventria noted that rice-derived proteins are not strong allergens and discussed RAST assays of subjects with rice allergies.
c. Ventria described differences in binding, including: (i) receptor binding in CACO-2 cell model, noting that differences (higher binding in rice-derived protein) may related to glycosylation; (ii) binding of bacteria (Enteropathogenic *Escherichia coli* (EPEC)) and reduction in their growth; (iii) rice-derived lactoferrin bound higher levels of iron. (Ventria noted that iron binding correlates inversely with bacteriostatic activity).

2. Production and purification
   Dr. Bethell described the procedure for production of the protein and discussed two possible products a concentrate which would contain rice proteins and an "isolate" which would be further purified.
3. Toxicology
   a. Dr. Bethell described the results of a 28-day study on rats of the protein which resulted in a NOAEL and discussed plans for a further study.
4. Proposed Uses and Levels of Use
   a. Ventria intends for its protein product to be use in iron fortification as per 21 CFR 170.3 (o) (20) in medical foods (oral rehydration solutions for pediatric and geriatric uses) and in bars and beverages at levels from 1-4 g/p/d. Ventria discussed that about 1 g per infant per day is consumed in breast milk.
   b. DBGNR representatives discussed that claims and use in medical foods will likely require consultation with representatives from appropriate units within the Office of Nutritional Products, Labeling and Dietary Supplements (ONPLDS).
5. Time frame
   a. Ventria expressed its plan to submit the notice during this calendar year.
   b. The parallel tracks of Ventria's current BNF and this putative GRN were discussed along with the need to keep upper management appraised of its progress and a possibility that the two notifications may need to be complete before closure of either.

Dr. Merker discussed the need for a discussion of "what makes it GRAS" in the submission; that is, that the weight of the evidence is on generally available information (published, preferably in peer-reviewed journals) rather than new unpublished studies. Dr. Bethell responded that there are many available publications about lactoferrin and its safety from multiple biological sources. Ventria planned to address the applicability of these publications to their studies showing that their rice derived protein was not significantly different.

Ventria left with final questions regarding the adequacy of the current and planned toxicology experiments and whether using a substantial equivalence model for general recognition was acceptable.

Robert I Merker

Robert I. Merker, Ph.D.

cc: Correspondence CTS 80062 SBJ 001366
electronic mail cc: HFS-255 (JDietz, FEshete, JFasano, REdelstein, JGGlew, PRice, RHarris, JMihalov, AMattia, RLMartin, MDiNovi, LLubin, LSKahl)
R/D: HFS-255: RIMerker: 4/26/04
Comments from : JGGlew and JDietz
Init: HFS-255: RHarris: 6/28/04
F/T: RIMerker: 6/28/04

Memorandum of Telephone Conversation

Date: July 14, 2005

Between: Paulette Gaynor, Ph.D. (HFS-255)
Robert Merker, Ph.D. (HFS-255)

and

Delia Bethell, Ph.D. (Ventria Bioscience (916-921-6148))

Subject: GRNs 000174 and 000162

Ventria Bioscience submitted an electronic copy of the subject GRAS notices in addition to the requisite paper copies. Our Imaging Center used these electronic copies as the basis for the electronic files uploaded in FARM.

Dr. Robert Merker and I telephoned Dr. Bethell for clarification about pages in Appendix B of the subject GRAS Notices. We were seeking clarification about the order of the pages in this appendix for both GRAS notices because the electronic copy of the pages within this appendix had a different order from the paper copy. We were also seeking clarification about an October 7, 2003, letter with Confidential on it in this appendix in GRN 000174. Later on this date, Dr. Bethell sent an electronic mail message clarifying that the paper copies can be reordered so the electronic and paper copies are identical, and that for GRN 000174, the letter in Appendix B marked confidential is not considered confidential business information (see attachment).

Paulette M. Gaynor, Ph.D.

Attachment - Electronic mail message of July 14, 2005, from Delia Bethell of Ventria Bioscience to Paulette Gaynor and Robert Merker of FDA.

cc: GRN 000162 and GRN 000174

drafted:HFS-255:PMGaynor:7/18/05
comments:HFS-255:RIMerker:7/19/05
f/t:HFS-255:PMGaynor:7/21/05

Gaynor, Paulette M

From: delia bethell [dbethell@ventria.com]
Sent: Thursday, July 14, 2005 3:20 PM
To: Gaynor, Paulette M; Merker, Robert I
Subject: Lysozyme GRAS

Dear Drs. Gaynor and Merker,

As per our phone conversation, there is no significance to the order of the pages in Appendix B of the GRN 000162 or GRN 000174. The paper copies can be reorder to so the electronic and paper copies are identical.

In GRN 000174, the letter in Appendix B marked Confidential is not considered confidential business information. It should be in both the paper and electronic copy,

If there are any questions, please don't hesitate to contact me.

Regards,

Delia Bethell

Delia R. Bethell, Ph.D.
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November 9, 2005

Laura M. Tarantino, Ph.D. (HFS-200)
Director, Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
Room 3044
University Station
4300 River Road
College Park, Maryland 20740

Re: Safety Concerns Raised by Recombinant Human Lactoferrin from Rice
(GRN No. 000162 Submitted by Ventria Bioscience)

Dear Dr. Tarantino:

Thank you for agreeing to meet with Agennix, Inc. on November 14, 2005 to discuss the claim of Ventria Bioscience ("Ventria") in GRN No. 000162 that recombinant human lactoferrin (rhLF) from rice is generally recognized as safe (GRAS) for use in functional foods, functional beverages, and medical food. Agennix is a biopharmaceutical company focused on developing protein-based drugs for the treatment of cancer and diabetic ulcers. We have significant experience with rhLF from a fermentation process that conforms to current good manufacturing process (cGMP) requirements for drugs, and have been conducting clinical trials with oral rhLF under Investigational New Drug Applications (INDs) filed with the FDA since 1996. Agennix recently completed blinded, placebo-controlled Phase II clinical trials with rhLF that met their primary efficacy endpoints in indications including non-small cell lung cancer and diabetic foot ulcers.

In anticipation of our meeting, we have carefully reviewed GRN 000162 and consulted with leading experts qualified by scientific training and experience to assess the safety of rice-produced rhLF for the proposed uses. As explained more fully below and in the attached scientific assessments, serious concerns and unanswered questions preclude any determination that rice-produced rhLF is GRAS. Indeed, opinions of qualified experts confirm that rhLF is a potent and complex bioactive molecule for which extensive investigations of appropriate size and duration—far beyond those described in GRN 000162—are warranted to establish safety. Accordingly, we respectfully ask that FDA conclude that this notification does not provide a basis for a GRAS determination. The scientific assessments on which this request is based are provided in **Attachments 1-3**. 1/

1/ **Attachment 1** provides a detailed assessment of safety concerns raised by the claimed GRAS status of rhLF from rice. **Attachments 2 and 3** provide additional views of Dr. E.D. Weinberg and Dr. Hubertus Schellekens, respectively.

I. THE GRAS STANDARD

As you are aware, a substance added to food is a "food additive" for which FDA pre-market approval is required unless the substance is GRAS or qualifies for another statutory exemption. The intended use of a substance is GRAS if it is—

> generally recognized, among experts qualified by scientific training and experience to evaluate its safety, as having been adequately shown through scientific procedures (or, in the case of a substance used in food prior to January 1, 1958, through either scientific procedures or experience based on common use in food) to be safe under the conditions of its intended use . . . 2/

As the statutory language suggests, a GRAS determination may be based either on "scientific procedures" or common use in food prior to 1958. A GRAS determination based on scientific procedures requires the same quantity and quality of scientific evidence as is required to obtain approval of a food additive regulation for the ingredient. 3/

Based on the statute, FDA has advised that a GRAS determination requires three elements:

1. Evidence that a substance is safe for its intended use;
2. A basis for concluding that such evidence of safety is generally available; and
3. A basis for concluding that such evidence of safety is the subject of scientific consensus among qualified scientific experts.

FDA refers to the first element as "technical evidence of safety"; the second and third criteria collectively constitute the "common knowledge" element of the GRAS standard.

Technical evidence of safety requires a showing that "there is a reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use." 4/ This is frequently paraphrased as demonstrating that there is a "reasonable certainty of no harm." The second element, general availability, requires publication of key data or information in peer-reviewed scientific journals, general reference materials, textbooks, or other appropriate sources. 5/ The third element, expert consensus, may be demonstrated by the scientific literature, documentation of the opinion of an expert panel, or the pertinent opinion of an authoritative body, such as the National Academy of Sciences (NAS), among other references. 6/ Expert consensus

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2/ FFDCA § 201(s).

3/ 21 C.F.R. § 170.30(b).

4/ 21 C.F.R. § 170.3(i); 62 Fed. Reg. 18937, 18948 (Apr. 17, 1997).

5/ 21 C.F.R. § 170.30(b); 62 Fed. Reg. at 18942-43.

6/ 62 Fed. Reg. at 18940-43.

does not require unanimity; however, the existence of a "severe conflict' among experts will preclude a GRAS determination.7/

II. APPLICATION OF THE GRAS STANDARD TO RECOMBINANT HUMAN LACTOFERRIN FROM RICE

Ventria fails on all three counts of the GRAS standard. Specifically, (1) GRN 000162 fails to establish that rice-produced rhLF presents a reasonable certainty of no harm under the intended conditions of use; (2) Ventria fails to cite any published studies that credibly support the safety of rhLF from rice; and (3) a severe conflict exists between scientists consulted by Ventria and numerous highly qualified scientists with specific expertise in lactoferrin, the toxicological significance of glycosylation, immunogenicity of recombinant proteins and other subjects pertinent to a thorough evaluation of GRN 000162.

Technical Evidence of Safety

As the attached scientific assessments state, rhLF is a complex molecule with potent biological activity for which a rigorous safety assessment is warranted. In a drug context, extensive clinical trials and post-market surveillance are needed to adequately assess the safety of a bioactive substance such as rhLF because adverse reactions may not be evident absent extended study. In a food context involving comparable conditions of use, an even greater assurance of safety is essential to account for the general availability of the product and absence of direct medical supervision.

In GRN 00162, Ventria asserts that rhLF from rice is GRAS for use in functional foods and drinks at levels not to exceed 1000 mg/serving and in medical foods at levels not to exceed 1 mg/mL. The assertion that rhLF from rice is GRAS is based on (i) claimed substantial equivalence between rhLF from rice and native human lactoferrin, rhLF from a cGMP fermentation process, and/or bovine lactoferrin, and (ii) opinions of an expert panel that rhLF from rice presents no immunogenicity, allergenicity, or other safety concerns. As described in the attached assessments, however, Ventria's analysis fails to adequately address numerous important safety issues, including the following:

- Differences of potential toxicological significance between rice-produced rhLF and other types of lactoferrin, including native human lactoferrin, bovine lactoferrin, and rhLF from a cGMP fermentation process.

  The glycosylation pattern that is unique to rice-produced rhLF is of particular concern. Glycosylation can have a significant impact on the function and safety of proteins, including impacts on pharmacokinetics, immunogenicity and allergic potential, stability, resistance to thermal or enzymatic degradation and specific activity.

- The complete absence of relevant studies sufficient to assess the safety of rice-derived rhLF.

7/ *See* 62 Fed. Reg. at 18939.

Only one clinical trial with rice-produced rhLF is referenced in GRN 000162—an unpublished study with four subjects who consumed rice-derived rhLF for six weeks. Other clinical trials cited by Ventria involved rhLF from a cGMP fermentation process, which differs significantly from rice-derived rhLF. The only published animal study cited by Ventria involving rice-produced rhLF was a pharmacology efficacy study that compared rhLF activity with that of antibiotics. This animal study was not designed to detect toxicity; it therefore is not reasonably relied upon to establish safety.

- Immunogenicity and allergenicity concerns.

  The GRAS submission does not adequately address (i) immunogenicity risks posed by major differences between the composition of rice-produced rhLF and native lactoferrin, (ii) the possibility that administration of rhLF with rice glycosylation, along with up to 10% contaminating foreign proteins and carbohydrates, may induce recognition of rhLF as a foreign protein with resulting cross-reactivity to an individual's native lactoferrin, and (iii) the potential immunogenicity of the unique protein sequence of the rice-produced rhLF in individuals with allelic variants.

- Potential induction or exacerbation of autoimmune disease and generation of anti-lactoferrin antibodies.

  Published literature cited in the GRAS Notice indicates that (i) lactoferrin is a potent immunostimulatory molecule known to induce a systemic immune response in both animals and humans, (ii) anti-lactoferrin antibodies are associated with a host of serious human autoimmune diseases, and (iii) there is animal evidence suggesting that lactoferrin might indeed exacerbate autoimmune disease. These concerning references are not adequately addressed.

- Other risks associated with extended dosing with any rhLF.

  These include risks of iron-overload in susceptible individuals, iron delivery to iron-constrained pathogens, iron delivery to tumors, systemic amyloidosis caused by lactoferrin variants, induced changes to immune function, induction of antibiotic resistance and viral activation.

- An intended daily dose far in excess of exposure to native LF (which is not equivalent to rhLF from rice in any event).

  The intended daily dose for rice-produced rhLF cited by Ventria is a thousand times higher than that resulting from the levels of native LF claimed to be present in saliva.

- Concerns relating to the manufacturing of rhLF in rice.

  Highly controlled systems for production, purification and characterization are required to ensure the integrity and safety of complex recombinant proteins. The production of rhLF in rice is not sufficiently controlled to allow for a consistent and

pure final product that is free from potentially harmful impurities, degradation products, and contaminants.

Based on these and other concerns, the experts consulted by Agennix found GRN 000162 to raise substantial issues and unanswered questions that preclude a finding of safety for the intended conditions of use. Indeed, these experts believe that rigorous testing, including clinical trials of appropriate size and duration, would be required before rice-produced rhLF could be considered safe for addition to the food supply.

This last point is particularly important. Although many GRAS determinations have been made, and should continue to be made, based on an established battery of animal toxicology studies and safety factors that establish safe conditions of use, there are some compounds that must necessarily be subject to rigorous clinical testing in order to demonstrate a reasonable certainty of no harm, as required by the statute. This especially includes molecules whose potent biological activities may not be accurately reflected in animal models, and particularly immunomodulatory molecules whose full spectrum of activity can only be observed following extended administration and surveillance in humans. We believe that rhLF, with its potent biological effects demonstrated in Phase II cancer trials and diabetic ulcer trials, is one such substance.

Past examples of compounds that also warranted significant clinical testing data include artificial sweeteners and fat substitutes, so FDA has ample precedent to require such significant clinical testing. Even though these other examples were in the context of a food additive petition, the legal standard for showing technical evidence of safety, as noted on page 2 above, is exactly the same for food additives and GRAS substances. We believe, as a matter of scientific evidence, that extensive clinical trials are needed for rhLF in order to adequately investigate the critical question of how humans will react to this compound under widespread conditions of long term use. The scant clinical evidence referenced in GRN 000162 does not even scratch the surface of what is needed to meet the statutory standard and protect public health.

The concerns stated above are serious and should preclude GRAS status for any functional food, functional beverage, or medical food application of rhLF from rice. These concerns are even more pressing in light of the therapeutic uses for which rice-produced rhLF is apparently intended. Public statements of Ventria and the GRN itself indicate that rhLF from rice will be expressly or implicitly promoted for therapeutic uses that are functionally indistinguishable from proposed drug uses undergoing critical evaluation by FDA's Center for Drug Evaluation and Research. In numerous statements as recent as October 2005, Ventria publicly described rhLF as a "pharmaceutical" and a "medicine" with potential to "treat disease" and "save lives." Ventria has even publicly stated its intention to use rhLF to treat diseases including inflammatory bowel disease (IBD), acute respiratory infections, and chronic lung infections caused by *pseudomonas* in patients with cystic fibrosis. A list of public statements by Ventria describing Ventria's intent to market rhLF as a medicine to treat disease is provided in **Attachment 4**.

We recognize that the regulatory classification of a product as a "drug" due to an intent to treat, prevent, cure, or mitigate disease does not typically factor into an assessment of whether the product meets the GRAS standard (i.e., assessment of whether a product is a "drug" under section 201(g) of the Federal Food, Drug, and Cosmetic Act is not usually considered as part of a

GRAS assessment conducted under section 201(s)). By law, however, the safety of a substance that will be added to food is to be assessed in light of its intended use, taking into account its "probable consumption." Based on Ventria's apparent intent to market rhLF from rice as a drug, it is entirely appropriate for the Center for Food Safety and Applied Nutrition (CFSAN) to consider the unique types of harms that may result from individuals consuming rhLF for perceived therapeutic effects in potentially unlimited doses for unlimited periods of time. Indeed, it appears to us that the law requires consideration of these factors and that a GRAS determination for rhLF should require a safety assessment even more rigorous than that required by CDER, to account for general availability of the substance without prescription or ongoing medical supervision.

The Common Knowledge Element—Publication

Ventria cites no published studies that support the safety of rhLF from rice. The only published study cited by Ventria involving rice-produced rhLF was a pharmacology efficacy study in animals that compared rhLF activity with that of antibiotics. This study was not designed to assess toxicity, and therefore is not reasonably relied upon to establish safety.

All other studies cited by Ventria are either unpublished or are not applicable to rhLF from rice, including studies conducted with rhLF from a cGMP fermentation process. Further, Ventria's assertions concerning the substantial equivalence of rhLF from rice to native human lactoferrin, rhLF from a cGMP fermentation process, and/or bovine lactoferrin are unfounded in light of published information to the contrary concerning such biologically important features as glycosylation and specific contaminants. Accordingly, GRN 000162 fails to satisfy the second element of the GRAS standard—demonstration that key studies and information supporting the GRAS determination are generally available to qualified experts.

The Common Knowledge Element—Severe Conflict Among Qualified Experts

Finally, Ventria fails to satisfy the third element of the GRAS standard—demonstration that the safety of the proposed use of rhLF from rice is the subject of expert consensus. Consensus is lacking because numerous experts qualified by scientific training and expertise to evaluate the safety of rice-produced rhLF do not consider it to be safe or generally recognized as safe for use in food.

As discussed in FDA's GRAS proposal and the pertinent case law, a proponent of a GRAS claim bears the burden of establishing expert consensus (i.e., that experts "generally" consider the ingredient at issue to be safe). The courts and FDA have interpreted this to mean that, although a mere divergence of views will not necessarily preclude GRAS status, as "even properly conducted studies may produce disagreement," 8/ a "severe conflict" of expert opinion will prevent a finding of general recognition. 9/

8/ *See, e.g., United States v. Articles of Food and Drug . . . "Coli-Trol 80"*, 518 F.2d 743, 746 (5th Cir. 1975).

9/ 62 Fed. Reg. at 18939 (citing *United States v. Articles of Drug . . . 5,906 boxes*, 745 F.2d 105, 119 n. 22 (1st Cir. 1984); *United States v. An Article of Drug . . . 4,680 Pails*, 725 F.2d 976, 990 (5th Cir. 1984);

There is no bright-line test for identifying what constitutes a "severe conflict," but courts have found a "severe conflict" to exist after evaluating the merits of each situation. In one case, even where the proponent of a GRAS claim presented the testimony of seven experts supportive of GRAS status, general recognition was found to be lacking in light of persuasive opposing views offered by "several" government experts. 10/ In another case, "sharply divided testimony" was found to present a severe conflict of opinion. 11/ Expert testimony critical of general recognition in that case suggested that the studies presented did not prove safety or efficacy and that the studies were not "well controlled" within the meaning of FDA's regulations. 12/ Although these and other cases addressing expert consensus involve drug products, the expert consensus standard is the same for both food products and drugs. 13/ For both food products and drugs, determining whether there is a meaningful and substantive dispute is key.

Expert credentials are also important when assessing whether expert consensus exists. In one case evaluating the status of a drug for the treatment of various vaginal infections, the court gave great weight to the opinions of several chairmen of leading Obstetrics and Gynecology departments. The court stated that "it cannot be denied that the affidavits of five of the leading doctors in the field which deny general recognition creates more than a 'mere' conflict . . . [i]t is inconceivable that a drug such as this could be considered generally recognized in the face of such learned non-recognition." 14/

Finally, the general quality of the evidence on which expert consensus is suggested to be based is also relevant. A lack of general recognition was found in one case where expert witnesses knew of no studies supporting a finding of general recognition and the manufacturer responded with "irrelevant or incomplete studies, expert opinions based on these tests or clinical experience (as opposed to clinical studies), and the interested opinions of . . . salesmen." 15/ The court stated that general recognition is precluded where there is a "lack of the proper reputation for ... safety

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Premo Pharmaceutical Lab. v. United States, 629 F.2d 795, 803 (2d Cir. 1980); *Coli-Trol 80*, 518 F.2d at 746 (5th Cir. 1975); *United States v. Articles of Drug . . . Promise Toothpaste*, 624 F.Supp. 776, 782 (N.D. Ill. 1985), *aff'd* 826 F.2d 564 (7th Cir. 1987)).

10/ *See, e.g., Pails*, 725 F.2d at 990 (holding that presentation by the United States of the views of "several experts" that a drug was not generally recognized as effective showed a "severe conflict" in the expert testimony and precluded general recognition).

11/ *United States v. An Article of Drug . . . X-Otag Plus Tablets*, 441 F.Supp. 105, 113-114 (D. Colo. 1977).

12/ *Id.* at 113.

13/ *See, e.g.*, 62 Fed. Reg. at 18938-18939 (citing drug and food precedent in discussion of meaning of GRAS standard under section 201(s) of the FFDCA).

14/ *United States v. An Article of Drug . . . "Mykocert"*, 345 F.Supp. 571 (N.D. Ill. 1972).

15/ *"Coli-Trol 80"*, 518 F.2d at 747.

of the food additive among appropriate experts" or "what reputation there is, is not based on adequate studies." 16/ Accordingly, even expert opinions lack persuasive value where the underlying evidence is weak.

Agennix, the clear worldwide leader in research, development and production of rhLF, has consulted the leading international experts on lactoferrin, glycosylation, immunogenicity, biosimilars and related subjects relevant to the safety of rhLF from rice. These experts include, among others, a pioneer in the field of lactoferrin research, a founder and director of a major center for Medical Glycobiology, and an author of more than 200 papers in peer-reviewed journals addressing biotechnology-derived therapeutic proteins (with a recent emphasis on biosimilars and the immunogenicity of therapeutic proteins). These highly qualified experts have expressed serious concern regarding the safety of rhLF from rice, demonstrating a "severe conflict" of expert opinion. Although the opinions of one or two of these experts would be compelling, the opinions of the numerous experts concurring in the attached scientific assessments unambiguously demonstrates a "severe conflict" that precludes GRAS status.

In summary, the clear lack of scientific consensus that rhLF is GRAS is evidenced by the published literature raising legitimate safety questions and by the views of scientific experts whose opinions are expressed in the attached scientific assessments. That so many, and such highly qualified, experts have expressed serious concern about the proposed use qualifies as a "severe conflict" of expert opinion and precludes GRAS status for rhLF from rice.

III. CONCLUSION

After carefully reviewing GRN 000162 and consulting with leading experts qualified to judge the complex safety issues raised by rhLF from rice, it is our view that Ventria presents insufficient data and information to reach any credible conclusion about the safety of rice-produced rhLF in humans. In view of the documented biologic activity of rhLF and its ability to induce clinically significant changes in immune function, we believe that rice-produced rhLF has not been shown to be safe for use in food products under the anticipated conditions of use. Accordingly, we ask that FDA respond to this GRAS Notice by concluding that an adequate basis for a GRAS determination has not been provided.

Agennix appreciates CFSAN's consideration of this important information as Ventria's GRAS exemption claim for rhLF from rice is considered. Please do not hesitate to contact us if there are any questions or if additional information would be useful.

Sincerely,

Rick Barsky
Chief Executive Officer

16/ *Id.* at 746.

Cc: Robert L. Martin, Ph.D. (HFS-255)
Supervisory Consumer Safety Officer
Division of Biotechnology and GRAS Notice Review

SAFETY CONCERNS RAISED BY RECOMBINANT HUMAN LACTOFERRIN PRODUCED IN RICE: SCIENTIFIC ASSESSMENT OF GRAS NOTICE NO. 000162 SUBMITTED BY VENTRIA BIOSCIENCE

We have been asked to review data and information presented in GRAS Notice No. 000162 concerning the safety of recombinant human lactoferrin (rhLF) produced in rice. This Notice asserts that rhLF from rice is generally recognized as safe (GRAS) within the meaning of the Federal Food, Drug, and Cosmetic Act for use in functional foods and drinks at levels not to exceed 1000 mg/serving and in medical foods at levels not to exceed 1 mg/mL. The assertion that rhLF from rice is GRAS is based on (i) claimed substantial equivalence between rhLF from rice and native human lactoferrin, rhLF from a cGMP fermentation process, and/or bovine lactoferrin, and (ii) opinions of an expert panel that rhLF from rice presents no immunogenicity, allergenicity, or other safety concerns.

RhLF is a complex molecule with potent biological activity for which a rigorous safety assessment is warranted. We are aware of Phase II clinical trials in which rhLF from a cGMP fermentation process was found to meet efficacy endpoints for indications such as non-small cell lung cancer. Due to the demonstrated biological activity of rhLF, extensive clinical trials and post-market surveillance are needed to adequately assess its safety in a drug context; in a food context involving comparable conditions of use, a greater assurance of safety is indicated to account for the general availability of the product and likely use without direct medical supervision. The conditions of use for rhLF from rice as described in the Notice are assumed to be comparable to likely drug uses because the identified "functional food" and medical food categories are consumed for perceived effects on bodily structures or functions (as opposed to technical effects in food processing), the rhLF will be recommended for consumption at comparable dosage levels, and the Notifier has made statements suggesting that the products will be marketed for express or implied benefits of a pharmaceutical nature.

In our expert opinion, the Notice raises substantial issues that preclude a finding of safety (i.e., a reasonable certainty of no harm) for the conditions of use and warrant further investigation, including clinical trials of appropriate size and duration. Of particular concern are the absence of adequate trials with rhLF from rice, differences of possible toxicological consequence between native human lactoferrin and rhLF from rice, and failure of the Notice to sufficiently address a wide range of safety risks, including risks of immunogenicity and allergenicity. We also note that the intended daily dose dramatically exceeds exposure from native lactoferrin. This assessment addresses the following specific concerns:

1. Absence of adequate safety studies conducted with rhLF from rice,
2. Risks specifically associated with the glycosylation of rhLF from rice,
3. Risks of immunogenicity and allergenicity with rhLF from rice,
4. Risks of rhLF exacerbating autoimmune disease,

5. Other risks associated with extended dosing with any rhLF, and
6. Significance of the intended daily dose of rice-produced rhLF.

We also note what appear to be substantial safety concerns relating to the manufacture of rhLF from rice as described in GRAS Notice No. 000162. These concerns are addressed in detail in **Appendix 1** and include the following observations:

- Characterization of rice-produced rhLF is incomplete and inadequate.
- Production and purification processes are not adequately controlled.
- Long-term stability data are absent for rice-produced rhLF.

For these reasons, we conclude that available information fails to establish rice-produced rhLF as presenting a reasonable certainty of no harm under the claimed or probable conditions of use. Specific data and information supporting this conclusion are presented in detail below.

1. Safety Studies with Rice-Produced rhLF

GRAS Notice 000162 presents minimal data from safety studies with rice-produced rhLF. The following tables summarize all of the studies conducted with rhLF cited in the GRAS submission.

Animal Studies with Notifier's RhLF

Species	(n=)	Duration	Dose	Reference	Location
Rats	N/A	28 Days	1000-1800 mg/kg/day	**(Unpublished)** Notifier's rhLF	Page 23,66
Rhesus Monkeys	N/A	120 Days	500-800 mg/kg/day	**(Unpublished)** Notifier's rhLF	Page 64
Broiler Chicks	660	N/A	Commercial rice animal feed containing 5% lactoferrin and 5% lysozyme	Humphrey 2002 (Notifier's rhLF) **(Not a Safety Study)**	Page 23

Human Studies with RhLF From Other Sources

Species	(n=)	Duration	Dose	Reference	Location
Humans	12	1 Day	250 mg X 5 doses (6 subjects), 1,000 mg X 5 doses (6 subjects),	Opekun 1999 **(Agennix rhLF)**	Page 24
Humans	9	5-14 Days	5 grams per day X 5 days (6 subjects), 5 grams per day X 14 days (3 subjects)	Guttner 2003 **(Agennix rhLF)**	Page 24

Humans	23	Bolus, 1 Day	5 grams bolus (8 Subjects), 3 X 5 grams in 24 hrs (15 subjects)	Troost 2003 **(Agennix rhLF)**	Page 25
Humans	33	14-28 Days	1.5, 4.5, or 9 grams per day	Hayes 2003 **(Agennix rhLF)**	Page 25
Humans	4	42 Days	4.8 grams per day	**Unpublished** (Notifier's rhLF)	Page 25

Notes: N/A = Not Available. Agennix rhLF is pharmaceutical grade, cGMP material produced by controlled fermentation. The glycosylation of rhLF produced by Agennix differs substantially from rhLF produced in rice.

The data presented in GRAS Notice 000162 are insufficient to substantiate the safety of rice-produced rhLF. All of the published human safety data referenced in the Notice utilize pharmaceutical grade rhLF produced by Agennix, Inc. using established fermentation techniques under cGMP conditions. These data are not relevant to rhLF from rice because substantial differences exist between rhLF from a cGMP fermentation process and rice-produced rhLF.

The GRAS submission references only one clinical trial with rice-produced rhLF. This unpublished study had only four subjects who consumed rhLF for six weeks. This study is not sufficient to establish the safety of a potent bioactive substance such as rhLF.

The one published study cited with rice-produced rhLF (Humphrey 2002) measured weight gain using rice feed in broiler chicks. This study was a pharmacology efficacy study that compared rhLF activity with that of antibiotics. It cannot, therefore, be used to support the safety of rhLF.

Although definitive guidelines still need to be finalized, there is a general opinion that even where products are derived from the same gene and the same host cells using identical down-stream processing, large side-by-side clinical comparisons are essential to establish whether recombinant proteins are similar (EMEA/CPMP/3097/02 Guidelines). This position has several implications for rice-derived lactoferrin:

- GRAS designation of a complex recombinant protein like rhLF is by definition difficult to establish because data derived from a specific product usually cannot be generalized to class level.
- Data not generated with a specific recombinant product as it will be marketed cannot typically be used to support the safety of such products.
- A market authorization of rhLF from a specific manufacturing process should be granted only on the basis of extensive safety testing of the product as it is manufactured for marketing.

For these reasons, published data with Agennix's rhLF cannot be relied upon to substantiate the safety of rhLF from rice. Furthermore, we are unaware of evidence

suggesting that rhLF from any source has been proven safe for general human consumption in unlimited quantities for unlimited duration.

2. Risks Specifically Associated With Glycosylation of Rice-Produced rhLF

On Pages 37-39 of the GRAS submission, it is acknowledged that native lactoferrin and rice-produced rhLF have different glycosylation patterns. The Notice further recognizes that glycosylation can play an important role in the function of biologically active molecules and discloses that two of the main glycans present on rice-produced rhLF are known allergens.

It is stated that α(1,3) fucose and β(1,2) xylose glycans appear on virtually 100% of rice-produced rhLF, and that these are cross-reactive carbohydrate determinants (CDDs) known to produce IgE antibodies. These same glycol-epitopes have been shown to induce immunogenicity in other therapeutic proteins produced in plants (Bardor 2002). It has been demonstrated that a considerable number of healthy individuals have antibodies circulating against α(1,3)-fucose and β(1,2)-xylose residues. Furthermore, these α(1,3)-linked fucose and β(1,2)-linked xylose glycans occur on many parasites and microorganisms that cause disease in people, and immune responses to these unusual carbohydrates, and even the carbohydrates themselves, may be profoundly important in disease pathogenesis (Die 2006, Foetisch 2003, Malandain 2005, Nyame 2004).

The reason for the allergic responses to plant-derived carbohydrate antigens is beginning to be better understood, and there is increasing concern about the safety of carbohydrate antigens of the type found on rice-produced rhLF. For example, a recent review notes that bee venom phospholipase A2 carries an N-glycan containing the α(1,3)-linked fucose, and several T-cell clones have been identified from bee venom-sensitized individuals that proliferate in response to honey bee PLA2 but not to its non-glycosylated variants, providing evidence for the involvement of N-glycans in T-cell recognition (Die 2006).

O-glycosylation is also relevant to safety and differs between plant and mammalian cells. In the latter, all amino acids with a hydroxyl group may be O-glycosylated while in plants this is mainly restricted to Hyp, Ser and Thr residues. The O-glycans of plants (including rice) are associated with a high level of immunogenicity (Showalter 2001). While the O-linked glycosylation of rhLF from rice has not been elucidated, it can be assumed to differ significantly from lactoferrin produced in mammalian cells.

The unnatural glycosylation of rice-derived rhLF can be expected to have important biological consequences. Glycosylation can have a significant impact on the function and safety of proteins, including impacts on pharmacokinetics, immunogenicity and allergenicity, stability and resistance to thermal or enzymatic degradation and specific activity.

There are many examples demonstrating the importance of glycosylation to the function of therapeutic proteins. For example, in monoclonal antibodies, glycosylation is

important for the Fc functions that determine clinical efficacy (Jefferis 2005). In proteins like erythropoetin, glycol-residues are necessary for receptor ligand interaction (Takeuchi 1991). The influence of glycosylation on pharmacokinetic behavior is illustrated by proteins such as aranesp, an erythropoetin with increased half-life due to the introduction of hyperglycosylation (MacDougall 2000).

Examples showing the effect of glycosylation on the immunogenicity of proteins are many (Schellekens [a] 2002). GM-CSF, a growth factor used to stimulate white blood cell differentiation and activity, is a case in point. When produced in E.coli, the protein is clearly more immunogenic than when produced in CHO cells. The glycosylation of GM-CSF covers epitopes that are exposed in non-glycosylated forms (Gribben 1990). In the case of interferon beta, the non-glycosylated protein forms aggregates that are strongly immunogenic (Karpusas 1998).

The GRAS Notice acknowledges that rhLF produced in rice has a high probability of triggering an IgE response due to the presence of the above-mentioned glycans: "The observations therefore demonstrate that replacing mammalian for plant N-glycans introduced IgE-recognition." The induction of IgE-recognition is not surprising, given the voluminous literature documenting the relationship between plant-derived carbohydrate antigens and allergic immune responses (Die 2006, Malandain 2005).

An attempt is made to explain away this danger by saying that IgE recognition does not necessarily lead to the generation of antibodies and, even if it did, "not all anti-IgE antibodies need to be involved in the generation of symptoms." To determine whether rice glycans might trigger histamine release, rice-produced rhLF was tested *in vitro* against serum derived from known pollen-allergic patients with anti-CCD IgE. It was concluded that moderate anti-CCD IgE titers in combination with rhLF carrying two reactive rice N-glycans does not result in histamine release.

This conclusion, however, is based on data from only three samples of serum. The sample size is clearly insufficient to draw any conclusion regarding IgE reactivity or safety. Furthermore, *in vitro* assays are inadequate for determining the safety of rice-glycosylated rhLF after oral administration in humans. *In vitro* assays are limited by the fact that plant-derived carbohydrate antigens on glycoproteins, such as rhLF, may not be provided in adequate form or quantity to cells used in the assay. *In vivo*, antigen-processing cells (APCs) modify glycoproteins by degradation into proteolytic and glycoproteolytic fragments. Such fragments would not be present in the *in vitro* assays described in the GRAS Notice.

Additional unsupported conclusions are offered: "In general, we can say that IgE antibodies against carbohydrate moieties have poor biological activity. Carbohydrates are not generally considered as allergens." These generalizations run contrary to the scientific evidence. The allergenicity of carbohydrate moieties has been documented and the statement is contradicted by a wealth of scientific publications (Breiteneder 2005, Andersson 2003, Die 2006).

In reality, carbohydrates are considered among the strongest antigens and allergens and many published studies document that the types of carbohydrates found on rice-produced rhLF are potent inducers of antibody responses, including IgE (Andersson 2003, Die 2006). Moreover, and most troubling, is the statement that carbohydrate moieties have poor biological activity. In fact, besides being antigenic and allergenic, carbohydrate moieties of glycolipids and glycoproteins in plants, animals and humans have very high biological activity and are involved in a tremendous range of biological processes including cell-cell adhesion, cell-cell signaling, immune regulation, and cell biological phenomena including organelle biosynthesis (Varki 1999). Thus, the presence of plant-derived carbohydrate antigens on rhLF raises the possibility that not only may they induce immune responses, but may in fact interfere with endogenous biology of human cells involving carbohydrate moieties.

Given that the role of carbohydrates as recognition elements in biology is well understood, there is no justification to ignore evidence that foreign glycoforms will have an effect on rice-produced rhLF's safety. Dismissal of the safety risks relating to glycosylation is improper for the following reasons:

1. Contrary to conclusions offered in the GRAS Notice, carbohydrate moieties have increasingly been implicated in the immunogenicity of recombinant proteins (Schellekens 2004, Hermeling 2004, Die 2006). Immunogenicity and antibody formation have been noted with insulin, interferon, epotin alfa and other recombinant proteins. Additionally, immunogenicity to recombinant proteins is independent of the route of administration. There have been no published cases where a change in route of administration completely negated immunogenicity (Schellekens 2003).

2. The Notice treats the glycans on rice-produced rhLF as food rather than structural components of a therapeutic protein vital to immune system recognition. RhLF is a biologically active immunostimulatory drug that interacts directly with receptors in the gut responsible for regulating immune response. Through receptor binding, lactoferrin might actually serve as a vector to deliver cross-reactive plant glycans directly to activated immune cells. It would seem likely that the presence of these glycans on lactoferrin might actually ***increase*** the risk of an IgE response. No studies have been done to assess this possible risk. Additional clinical studies are needed to assess these risks before any broad safety claims can be made.

3. The emergence of an antibody response and the breaking of B-cell tolerance require prolonged exposure to a recombinant protein, and generally antibodies can appear up to one year after chronic treatment (Schellekens 2004). The induction of anti-lactoferrin antibodies in people receiving exogenous lactoferrin has been documented (Brock 1998) and anti-lactoferrin antibodies have been associated with serious autoimmune disease. The induction of anti-lactoferrin antibodies could have tremendous consequences by "neutralizing" many other vital functions of ***endogenous*** lactoferrin, which is a degranulation product of neutrophils involved in, among other things, the regulation of inflammation.

Until robust studies are conducted to determine the effects of long-term exposure to rice glycans delivered by rhLF to immune cells in the gut, no general conclusions can be reached about the safety of rice-produced rhLF as described in GRAS Notice 000162.

3. Risks of Immunogenicity and Allergenicity

Recombinant therapeutic proteins are increasingly being found to carry long-term health risks related to immunogenicity and allergenicity. Rice-produced lactoferrin raises safety concerns in both of these areas.

3.1 Immunogenicity

The immunogenicity of therapeutic proteins can be based on two different mechanisms: the induction of antibodies by the presence of foreign epitopes and, the breaking of B-cell tolerance (Schellekens [b] 2002).

In the case of rice-produced rhLF, the presence of foreign epitopes is dependent on the structural differences between native human lactoferrin and lactoferrin produced in rice. The gene introduced in rice was chemically synthesized based on a lactoferrin sequence in the public domain. Multiple changes in the gene were introduced to facilitate efficient translation in rice. Peptide analysis provided in the GRAS submission shows major differences in the amino acid composition of rice-produced lactoferrin compared with the predicted sequence of the native gene. These changes present clear immunogenicity risks.

It is also concerning that the population frequency of the allele used to produce rhLF in rice is not disclosed. It is recognized that a substantial segment of the human population has variations in the lactoferrin gene (Liu 2002, Panella 1991), and that variant alleles can be associated with significant differences in biological activity (Velliyagounder 2003).

For such individuals, rice-produced rhLF may be recognized as a foreign, potentially immunogenic protein with resulting cross-reactivity to the individual's native lactoferrin. The GRAS Notice presents data showing extensive differences between rice-produced rhLF and native lactoferrin including glycosylation, molecular mass, isoform pattern and receptor binding. The serious health risks potentially arising from these differences are not addressed.

For the induction of antibodies and breaking of B-cell tolerance, the presence of impurities and contaminants is highly relevant (Schellekens [a] 2002). Because batch specifications for rice-produced rhLF allow 10% contaminants and impurities, immunogenicity related to these impurities cannot be excluded. Whatever mechanism leads to the induction of antibodies, immunogenic effects historically appear only after a *minimum* of 6 months exposure and may not appear for years in some cases. Long-term studies are therefore the only credible way to identify and quantify health risks associated with immunogenicity.

The potential immunogenicity of rice-produced rhLF is a serious issue because the induction of anti-lactoferrin antibodies:

- may be involved in autoimmunity,
- may cross-neutralize endogenous lactoferrin with negative biological effects, and
- may neutralize the efficacy of exogenous lactoferrin and compromise individuals for future treatments.

3.2 Allergenicity

Contaminants in rice-produced rhLF need to be carefully considered when evaluating its allergenic potential. According to batch release specifications provided in the GRAS Notice, rice-produced rhLF can contain up to 10% contaminants and impurities. A major fraction of these contaminants will consist of host-cell proteins making the allergenicity of the rice used to produce rhLF a serious concern. Introduction of the lactoferrin gene in rice plants will alter protein expression resulting in the activation and down regulation of a number of other genes. This effect potentially raises the allergenicity of the host rice plants.

The potential allergenicity of rice-produced lactoferrin itself is also a safety issue. Although it is still impossible to predict the allergenic behavior of a protein from structural data, lactoferrin has some characteristics that are shared by food allergens (Breiteneder 2005). These characteristics include binding of metal ions, interaction with membrane proteins, the presence of intramolecular disulphide bonds, N-glycosylation and resistance to heat denaturation.

In contrast to the arguments presented in the GRAS submission, there is good evidence that exogenous lactoferrin can cause allergenic responses. Lactoferrin is reported to have a stimulatory effect on delayed type hypersensitivity (Kruzel 2002, Kocieba 2002). In a recent study of patients with sensitivity to cow milk, 50% of the patients appeared to be sensitized by lactoferrin (Natale 2004). Furthermore, the plant glycan residues present on rice-produced rhLF are constituents of the glycol-epitopes of plant allergens showing IgE binding and causing mediator release by human basophils (van Ree 2000, Westphal 2003).

The GRAS Notice acknowledges that allergenicity is a relevant safety issue with rice-produced rhLF, yet concludes "the possible allergic property of a recombinant lactoferrin product can never be a reason to come to a negative verdict (*on GRAS Status*)." We strongly disagree with this conclusion. Known allergenic/ immunogenic properties are a significant safety concern and certainly should be a basis for questioning GRAS status. The biotechnology industry has developed a host of expression systems to make recombinant glycoproteins precisely to avoid introducing antigenic carbohydrates on the recombinant glycoproteins. The Notifier's planned use of rhLF and their dismissal of anti-carbohydrate responses is directly contradicted by a long history of safe production

of human glycoproteins in expression systems that minimize or avoid complications of non-human carbohydrate structures.

4. Risks of Exacerbating Auto-Immune Disease with rhLF from Rice

Another danger of long-term administration of rhLF in humans is the potential induction or exacerbation of autoimmune disease and the generation of anti-lactoferrin antibodies. On Pages 62-64 of the GRAS submission, explanation of these risks is initiated by acknowledging a substantial list of disconcerting references showing that anti-lactoferrin antibodies are often present in patients with autoimmune disease:

- Anti-lactoferrin antibodies have been associated with autoimmune liver disease (Ohana 1998).
- Anti-lactoferrin antibodies have been associated with inflammatory bowel disease (IBD) (Roozendaal 1999).
- Anti-lactoferrin antibodies have been associated with Wegener's granulomatosis (van der Woude 1985).
- Anti-lactoferrin antibodies have been associated with rheumatoid arthritis (Locht 2000).
- Anti-lactoferrin antibodies have been associated with systemic lupus erythaematosus (Galeazzi 1998).
- Anti-lactoferrin antibodies have been associated with autoimmune pancreatitis (Okazaki 2000).

The GRAS Notice admits that, in view of this evidence, it is important to determine a) whether ingestion of rhLF in humans produces anti-lactoferrin antibodies, and b) whether such antibodies have pathological significance. Certainly, the presence of highly unusual carbohydrate antigens on rice-produced rhLF raises the troubling possibility that the carbohydrates may in fact stimulate immunity to the protein itself, thereby generating neutralizing and/or inhibitory antibodies that could block functions of the protein. In fact, conjugation of such antigenic carbohydrates to so-called carrier proteins is the modern way to induce protective immunity to parasites and bacteria carrying unusual carbohydrates. This is well documented in a recent review citing specific examples of vaccine development using carbohydrate-conjugates to proteins (Nyame 2004).

The GRAS Notice cites evidence suggesting that administration of bovine lactoferrin in mice can produce a systemic immune response (Debbabi 1998, Sfeir 2004), and acknowledges that oral administration of human milk proteins containing 40% lactoferrin resulted in the production of IgG, IgM, IgA, and anti-hLF antibodies with spleen sensitization (Yuki 1998). Without presenting data, the Notifier dismissed these results by hypothesizing that this reaction was a response to a foreign antigen in these species. This unsupported conclusion is relied upon despite the fact that another referenced study

showed that ingestion of human lactoferrin by breast-fed human infants produced IgG and anti-hLF antibodies in those subjects (Brock 1998).

The primary evidence that rice-produced rhLF will not elicit a similar immune response is based on a single unpublished human safety study conducted by the Notifier in which only four adult women were administered 4.8 grams of rice-produced rhLF per day for 6 weeks. The data showed no evidence of the development of anti-lactoferrin antibodies. In spite of the previously cited studies clearly showing potential risk, the GRAS Notice concludes, presumably on the basis of this single study involving just four subjects, that the ingestion of human lactoferrin by healthy adults will not induce an autoimmune response. Any conclusion based on such minimal data is unwarranted and lacks credibility.

In view of the evidence presented demonstrating that lactoferrin can induce an immune response in animal models, as well as in human studies involving infants, the position taken in the GRAS Notice is unsupportable. Data are not presented on the impact of rice-produced rhLF in non-healthy subjects, who would also likely consume rhLF from rice if FDA does not question the GRAS petition. Nor does the Notice address the potential impact on patients with conditions that are known to be associated with anti-lactoferrin antibodies (autoimmune liver disease, inflammatory bowel disease, Wegener's granulomatosis, rheumatoid arthritis, systemic lupus and autoimmune pancreatitis). Some mention is made of potential risks of rice-produced rhLF, but credible evidence to mitigate the known safety concerns relating to autoimmunity is not presented.

Regarding the pathological significance of anti-lactoferrin antibodies, almost no evidence is presented contradicting the known risks. The GRAS Notice acknowledges a study claiming that the presence of anti-lactoferrin antibodies may be associated with inflammation of the colon (Roozendaal 1999). In a mouse model of rheumatoid arthritis, collagen-induced arthritis was exacerbated in transgenic mice expressing human lactoferrin (Guillen 2002). In spite of the fact that these two examples exacerbate safety concerns, this represents the extent of Notifier's data concerning the pathological significance of anti-lactoferrin antibodies. As indicated above, administration of rice-produced rhLF might induce anti-lactoferrin antibodies due to the carbohydrates acting as adjuvants. Thus, there is a real concern about the potential complications of oral administration of high amounts of rhLF containing plant-derived carbohydrate antigens.

The GRAS Notice concludes: "While there is evidence that orally-ingested lactoferrin can induce a systemic immune response and that antibodies to lactoferrin could theoretically be involved in disease progression, there is no evidence that the former can give rise to the latter." It further states, "It thus seems highly unlikely that ingestion of human lactoferrin as a food supplement, even in individuals with ongoing autoimmune disease, would have any adverse effect."

These conclusions relating to autoimmune disease are unsupported by any scientific data relating to rice-produced rhLF. What the arguments do show is that rhLF may impact

autoimmune disease. Contrary to showing that rhLF is GRAS for mass consumption, the facts, as revealed in published literature cited in the GRAS Notice, indicate that:

1) Lactoferrin is a potent immunostimulatory molecule that has been shown to induce a systemic immune response in both animals and humans, including the induction of anti-lactoferrin antibodies;

2) Anti-lactoferrin antibodies are associated with a host of serious human autoimmune diseases;

3) There is animal evidence that lactoferrin might indeed exacerbate autoimmune disease.

No data are presented to rebut these safety concerns. An unpublished, non-statistically significant "safety" study in four subjects cannot be considered indicative, much less conclusive.

Despite the known association of lactoferrin antibodies with serious human autoimmune diseases, the GRAS Notice fails to address the potential consequences of long-term consumption of rice-produced rhLF in people with these diseases. Moreover, while the potential risk of administering rhLF in a high dose oral bolus is acknowledged, no data are presented on the effects of such administration in humans. Given the documented risk of rhLF-induced autoimmune reactions, a conclusion of safety broad enough to authorize mass consumption by the general population in unlimited doses without medical supervision can only be based on properly controlled, definitive, long-term clinical trials in humans. No such trials are included in the GRAS Notice.

5. Other Risks Associated with Extended Dosing with Any RhLF

There are numerous other potential risks associated with the consumption of pharmacologically relevant doses of rhLF for extended periods of time by the general population, without any clinical surveillance.

5.1 Toxicity in Individuals with Iron Overload

As articulated in a standard hematology textbook (Hoffman 1998), "Iron overload denotes an excess in total body iron resulting from an iron supply that exceeds requirements. Because requirements are limited and humans lack a physiological means of excreting excess iron, any sustained increase in intake may eventually result in accumulation of iron. … When the accumulation overwhelms the body's capacity for safe storage, potentially lethal tissue damage is the result." The most common form of iron overload in the US is hereditary hemochromatosis, occurring in as much as 0.5% of the population or as many as 1 million individuals (Edwards 1993, Edwards 1988). Other forms, which also affect thousands of patients with substantially higher prevalence within specific population subgroups, include thallasemias and refractory anemias. Iron overload may also follow increased absorption of dietary iron in some patients with chronic liver disease, including those with alcoholic cirrhosis and portacaval shunting

(Jakobvits 1979). Iron overload can proceed asymptomatically for years, with the patient often presenting only after severe tissue damage has already occurred. Liver disease is the most common complication of iron overload resulting in hepatomegaly, functional abnormalities, fibrosis and eventually cirrhosis (Scheur 1962). Hepatocellular carcinoma can be an ultimate complication. Diabetes mellitus is a common complication of all forms of systemic iron overload (Stremmel 1988) occurring in 48% of patients. A raft of other complications have been reported including such fatal ones as iron-induced cardiac disease causing cardiomyopathy with heart failure, arrythmias or both (Model 1984).

The daily requirement of iron is only ~ 1 mg in adult men and ~ 2 mg in pre-menopausal adult women. In hereditary hemochromatosis, dysregulation of intestinal iron absorption occurs, wherein iron continues to be efficiently absorbed even in the face of substantial elevation of body iron stores eventually leading to the major morbidities and mortalities associated with the disease. Treatment for patients diagnosed with iron load disorders involves iron chelation by either regular phlebotomy or with chelating agents. Patients are also counseled to avoid foods rich in iron and avoid any iron containing supplements. The dietary concern is of course even more critical in the substantial number of patients with undiagnosed disease.

As described in their GRAS application (Section C.1, page 18), Notifier's "as is" lactoferrin contains 0.7-0.8 mg iron/gram lactoferrin. A projected daily adult consumption of 1-2 grams/day of lactoferrin (Table 6) thus translates into a daily iron supplementation of 0.75 to 1.5 mg of additional iron/day. This amount, which is comparable to the TOTAL daily iron requirement for an adult male, is provided in a highly bioavailable form whose long-term consumption would pose a very significant and unnecessary safety risk.

5.2 Iron Delivery to Iron Constrained Pathogens

Lactoferrin binds iron with a high avidity across a broad range of pH concentrations and its ability to deliver iron is an important biological property of this molecule. In experiments with human duodenal mucosa, unlike serum transferrin and ovotransferrin, lactotransferrin was able to yield its iron to intestinal tissue in a receptor-mediated process (Cox 1979). As has been discussed in the literature as a potential concern, administration of lactoferrin with its highly bioavailable iron can accelerate growth by pathogenic bacteria and protozoa (Weinberg 1978). An outstanding example of this involves infections caused by the enteric pathogen, *Vibrio vulnificus*, most often acquired by eating raw shellfish. When ingested by humans with iron-overload, this organism can cause rapidly progressing and fatal bacteremia (Wright 1981). In mice, the LD^{50} for an inoculum of *V. vulnificus* drops from 10^6 in normal mice to an estimated 1.1 organisms in iron-loaded animals, an impressive 6 log difference. Similar 5 to 6 log differences also have been reported for certain strains of *E. coli* (Eaton 1982). In humans, trauma-associated sepsis, which has often been linked to the ability of otherwise normal commensal bacteria to invade and penetrate the gut mucosal barrier, appears to involve catecholamine mediated iron removal from lactoferrin and its acquisition by bacteria (Freestone 2002).

Microbial colonies tend to be iron constrained (Andrews 2003), and access to a source of iron can induce infectious flare-up. In fact, a variety of bacteria have evolved a mechanism for acquiring iron directly from human lactoferrin. This mechanism involves surface receptors capable of specifically binding LF from the host, removing iron and transporting it across the outer membrane. The iron is then bound by a periplasmic iron-binding protein, FbpA, and transported into the cell via an inner membrane complex comprised of FbpB and FbpC (Elkins 2004). Iron availability is also critical to the virulence of *M. tuberculosis* and other mycobacteria that have also evolved a mechanism to acquire iron from lactoferrin (Ratledge 2004), as well as a variety of other pathogenic organisms.

The importance of iron levels in regulating bacterial growth is best expressed in the words of an editorial comment in (Shock 2002): "We all carry around a dangerous sack of goods—intestines filled with so many bacteria that they actually outnumber our own somatic cells. A dab of these lively intestinal contents released into the body is sufficient to kill any of us. For this reason, the guts are a site of constant and vigilant surveillance. … The aerobes … require iron and this requirement is their Achilles' Heel and, in some cases, our major protection. This is because iron is an exceedingly scarce commodity in normal mammalian body fluids… Some crafty bacteria have evolved iron-binding siderophores, such as desferrioxamine or enterobactin, which enable them to steal iron from normally safe iron reservoirs, in some cases even from the iron-binding proteins, transferrin and lactoferrin. Once this theft has occurred, bacterial growth is enabled and, clinically speaking, it is Katy, bar the door."

5.3 Iron Delivery to Tumors

The role of iron as a growth-regulating factor applies more broadly beyond micro-organisms. The growth of tumors is also known to be iron regulated (Weinberg 1983), and increased dietary iron has been shown to promote colon tumors in mice (Ilsley 2004, Hann 1991). Tumor cells are also known to over-express receptors that bind lactoferrin with a high affinity. These lactoferrin receptors have been shown to be up-regulated in the presence of iron chelators and to deliver iron to the interior of colon carcinoma tumor cells (Mikogami 1995). Thus, there is a risk that pre-cancerous or early stage GI tumors could also access iron from lactoferrin to accelerate their growth and metastasis.

5.4 Risks of Systemic Amyloidosis Caused by Lactoferrin Variants

In recent studies, lactoferrin variants have been linked to systemic amyloidosis. Amyloidosis is an acquired or hereditary disorder related to protein folding. Fragments of proteins that are normally soluble are deposited extracellularly where they accumulate and form deposits that interfere with the function of effected tissues or organs (Pepys 2001). These amyloid deposits have been implicated in the pathogenesis of diseases such as Alzheimer's disease, various prion diseases and type II diabetes.

Lactoferrin fragments have specifically been implicated as a cause of amyloidosis accompanied by trichiasis (a common vision threatening condition of the eyelid). The lactoferrin fragment responsible results from a single change from glutamic to aspartic acid near the end of the protein molecule (Ando 2003). The association of lactoferrin

fragments with amyloidosis is of particular concern because all recombinant forms of lactoferrin will contain variants and protein fragments that cannot be fully characterized or isolated given current technology (see **Appendix 1** on production issues). Given that the cited production standards for rice-produced rhLF are already far below cGMP pharmaceutical norms, adequate assurance that potentially pathogenic mutant proteins are not present cannot be provided.

5.5 Induced Changes to Immune Function

Studies show that, when administered in doses as large as those proposed by Notifier, lactoferrin can induce a change in immune system function (Zimecki 2001), including the induction of a TH1 shift. Transgenic mice over-expressing lactoferrin show a prominent TH1 immune shift (Guillen 2002). *In vitro* studies with lactoferrin indicate that it suppresses IL-4 and IL-10 production in respiratory epithelial cells obtained from human patients (Abraham 1992). *In vivo* rodent studies show that orally administered lactoferrin is a potent stimulator of IL-18 production in the gut, and is thus a stimulator of IFN-g production (Kuhara 2000, Varadhachary 2004). Enhanced IFN-g production is associated with the induction of a TH1 immune response.

Because of this potential to inhibit IL-4 and IL-10 and stimulate IL-18 / IFN-g, which shifts the immune balance from a TH2 to TH1 response, lactoferrin represents a potential risk for people with serious TH1-associated diseases like multiple sclerosis, type 1 diabetes and chronic obstructive pulmonary disease (COPD), among others.

It has also become clear that oral lactoferrin exerts a potent immunostimulatory effect including the production of key cytokines in the gut and in systemic circulation, an increase in circulating CD4+ and CD8+ cells and an increase in NK cell activity (Varadhachary 2004). These biological effects are important for pharmacological applications of lactoferrin (including the treatment of cancer). However, the long-term effect of chronic immunostimulation, including the possible induction of autoimmune diseases, is not known. There have been several recent examples of serious adverse events resulting from long-term administration of immunomodulatory agents, including most recently Tysabri (Drazen 2005).

5.6 Induction of Antibiotic Resistance

Another of lactoferrin's biological properties is its ability to interact with microbial membranes resulting in a variety of effects including depolarization. A recently published study demonstrated that exposure to rice-produced rhLF can induce antibiotic tolerance in *Pseudomonas aeruginosa*, an important pathogen responsible for numerous hospital infections (Andres 2005). Other negative bacteriological effects may also be associated with lactoferrin. For example, it was recently described that exposure of pathogenic streptococci to lactoferrin results in the induction of the streptococcal pyrogenic exotoxin A (Kansal 2005).

5.7 Viral Activation

Oral lactoferrin may be involved in viral transmission and the facilitation of viral replication. Lactoferrin has been shown to facilitate replication of HTLV-I by up-

regulating viral expression (Moriuchi 2001). Human lactoferrin in saliva has also been shown to act as a ligand for HHV-8, which suggests that orally administered rhLF could serve as a carrier for viral particles (Grange 2005).

5.8 Non-Equivalence with Bovine Lactoferrin

An attempt is made to support the safety of rice-produced rhLF by asserting its safety equivalence to bovine lactoferrin. However, there is no reason to believe that bovine lactoferrin and recombinant human lactoferrin are functionally equivalent. On the contrary, bovine lactoferrin has completely different glycosylation than native human or rice-produced rhLF (Wei 2000). Bovine lactoferrin also has a very different amino acid sequence with only 68% homology to human lactoferrin (Nuijens 1996), and is structurally different on X-ray crystallography (Sharma 1998). Given that 32% of the amino acid sequence is different and that the glycosylation is different, safety data with bovine lactoferrin is clearly no substitute for safety data with rice-produced rhLF. Further, published studies have demonstrated that bovine and human lactoferrins can have very different biological activities. For example the molecules differ in their core iron binding properties, and in experiments done by the same lab, bLF was found to act as an anti-angiogenic agent while hLF was pro-angiogenic (Norrby 2004, Norrby 2001). The biologically active N-terminal lactoferricin portions of the two molecule are even more divergent in terms of sequence, structure and function (Hunter 2005). Repeated assertions of rhLF's functional and safety equivalence to bovine lactoferrin, despite the substantial structural and functional dissimilarities, are scientifically unfounded.

6. Notifier's Intended Doses of rhLF

The intended daily doses of rice-produced rhLF are very high – in the same range as used (with cGMP fermentation produced rhLF) successfully in a recently completed Phase II clinical trial in non-small cell lung cancer. The GRAS Notice states that rhLF from rice is intended for use as follows:

In Foods:	0.7 – 1.0 grams per day
In Medical Foods:	0.2 – 2.2 grams per dose

The GRAS Notice proposes using a bolus of rice-produced rhLF that results in a daily dose that is a *thousand* times higher than that resulting from the 3.4 to 11.0 μg/mL levels of native lactoferrin that Notifier claims are consumed in saliva (Lentner 1981). Such large doses of rice-produced rhLF have never been safety tested in humans, either in Notifier's intended indications or for extended periods of time. The pharmacological effects of large doses of human lactoferrin are not fully understood and could pose health risks, including those related to significant and sustained changes in immune function.

The GRAS Notice does not present adequate data to address this potential safety risk.

Conclusion

There is a lack of scientific consensus regarding the long-term safety of rice-produced rhLF. Arguments and data presented by Notifier's GRAS submission highlight—rather that eliminate—known safety concerns. From published data, including data referenced in the GRAS Notice, the following may be reasonably observed:

- Proposed doses of rhLF are far in excess of those naturally occurring in saliva. Numerous published studies and recent clinical trials show that, in large doses, rhLF has a potent immunostimulatory effect. Notifier has neither conducted studies nor published data showing that large doses are safe in humans. Studies with Agennix's cGMP fermentation produced rhLF, which has completely different glycosylation, do not show the safety of rhLF produced in rice.

- Published data show that the main glycans present on rice-derived lactoferrin can induce a systemic immune response, including the generation of IgE antibodies in both animals and humans. IgE mediated immune responses are a serious health risk and, in extreme cases, can lead to anaphylaxis, or even death. Furthermore, the plant-derived carbohydrate antigens on rice-produced rhLF are also found on human parasites and could alter protective immune responses to such parasites.

- According to published studies, IgE antibody responses and the breakdown of B-cell tolerance can take up to a year of chronic exposure to an allergen to develop, making short-term clinical trials completely inadequate for detecting induced allergic sensitization or antibody development. Human exposure to rice-produced rhLF has been extremely limited to date and there is no clinical data whatsoever on the effects of long-term consumption.

- Anti-lactoferrin antibodies are known to be associated with, and potentially exacerbate, a wide range of serious human autoimmune diseases. Furthermore, rhLF is known to induce a potent TH1 immune response. No studies have been conducted to determine the consequences of long-term immunostimulation by rhLF in people with autoimmune disease.

Additionally, since lactoferrin is believed to directly interact with immune cells in the gut-associated lymphoid tissue, it is possible that, through receptor binding, rice-produced rhLF may act as a vector to deliver allergenic rice glycans directly to immune cells in gut associated lymphoid tissue. Lastly, there are other potential risks, as described above, associated with long-term administration of any rhLF, particularly in compromised patient populations.

Rice-produced rhLF presents numerous documented risks. These risks should not be casually dismissed—they should be thoroughly and scientifically evaluated. The potent immunostimulatory activity of rhLF warrants large, controlled, long-term clinical safety

studies before broadly exposing the public to potentially unlimited consumption. In fact, given the potential effects on lactoferrin-associated autoimmune diseases and the long period required to develop antibodies, rhLF from any source should be administered only under medical supervision. In our opinion, to expose the general public to these well-documented risks, without credible clinical safety data on the prolonged use of rice-produced rhLF, is both reckless and unnecessary. The designation of rice-produced rhLF as GRAS is inappropriate until it has been shown that the known risks (including, without limitation, the risks described in this scientific assessment) pose no threat to public safety.

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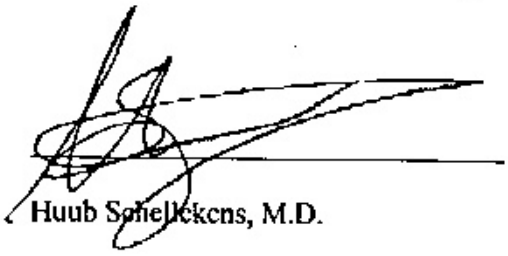
SAFETY CONCERNS RAISED BY RECOMBINANT HUMAN LACTOFERRIN PRODUCED IN RICE: SCIENTIFIC ASSESSMENT OF GRAS NOTICE NO. 000162 SUBMITTED BY VENTRIA BIOSCIENCE

The preceding scientific assessment of safety issues concerning rice-produced recombinant human lactoferrin has been provided by and reflects the opinion of:

Signature:  Nov 8, 2005

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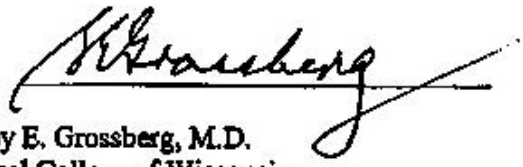
Signature: 

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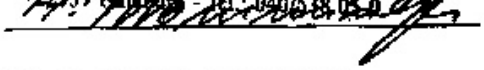
Signature: 

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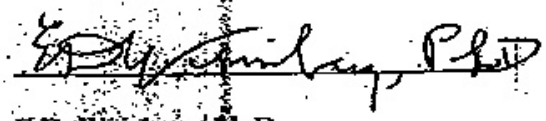
Signature: 

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Appendix 1

Production and Purification Issues

As the patents on the first generation of biotechnological protein drugs have started to expire, a debate has begun concerning the comparability of products produced by different manufacturers. Lactoferrin is a complex protein. In contrast with small molecules, analytical tools are not available for the full characterization of such proteins. It is also increasingly recognized that the biological properties of a protein product are highly dependent on the process of production. Consequently, efficacy and safety cannot be established by physical and chemical characterization and should always include data obtained from studies in humans.

Complex proteins like lactoferrin differ from small molecules in that they never consist of a single type of molecule, but are always heterogeneous mixtures of different isoforms and other variants caused by clipping, mis-folding, etc. Complex proteins are also relatively unstable. During storage, modifications such as oxidation, de-amidation and aggregation are common. These modifications can have a major effect on product efficacy and safety. To preserve the integrity of a recombinant protein, cold chain controls from manufacture to application are essential.

Physical chemical characterization

Section V of Notifier's GRAS submission gives details concerning the physical and chemical properties of rice-produced lactoferrin. The data presented, however, was generated during the development of the product and there is no detailed characterization of Notifier's rice-produced rhLF as it will eventually be marketed. It cannot be assumed that the biological characteristics of Notifier's rhLF will not change during production scale-up and transition from development to final manufacture.

The efficacy and safety of biologic proteins are highly dependent on the manufacturing process. Both FDA and EMEA (European Medicine Evaluation Agency) have established detailed guidelines for the evaluation of recombinant proteins following manufacturing changes. Notifier's manufacturing and quality control processes are rudimentary and inadequate for a proper evaluation of complex protein characteristics, including biologic activity. Major flaws in Notifier's characterization processes include the lack of amino acid sequencing, lack of glycosylation pattern identification and the failure to characterize isoforms, contaminants and impurities. Notifier fails to employ adequate testing to assure protein integrity. Required but missing testing methodologies include IEF, reversed phase HPLC, Western blotting, MALDI-TOF, SDS-PAGE and glycosylation analysis. Additionally, Notifier has not performed any biological characterization of the product.

This completely inadequate characterization of rice-produced rhLF precludes any use as food additive and makes a thorough safety evaluation of the product impossible. This in itself is sufficient reason to refuse marketing authorization and to deny GRAS status.

Production controls

Notifier's production controls are inadequate in several respects. To ensure uniform quality, the production of recombinant proteins needs to be strictly controlled. The manufacturing process should be described in detail and standards for all intermediate products must be met. Notifier's production process does not meet cGMP standards, which are considered essential for recombinant therapeutic proteins. No final product specifications are provided and impurities may be as high as 10%. Essential assays for batch release are missing, including a bioassay, which is critical to evaluate the potency of a biological product like rhLF. Batch release specifications should also quantify the levels of protein aggregates and lactoferrin variants (oxidation, clipped forms etc). None of these production controls have been adequately defined or established by Notifier.

Furthermore, Notifier's rice-produced lactoferrin is not aseptic, which is unusual for recombinant proteins. Microbial contamination is therefore a significant risk. Batch release specifications should also include a test for endotoxins and an assay for viral contaminants. These production risks have not been addressed.

Long term stability

Notifier provides no data on the stability of lactoferrin in stored rice, manufactured intermediates or the final product. Stability studies are essential not only to support any claim of biological activity but also to exclude the presence of harmful degradation products that have been widely described to occur in recombinant proteins during storage. Long term stability testing is particularly vital in Notifier's case because production and storage are not under sterile conditions and because a cold chain is not strictly controlled. Without these controls, there is a significant risk that microbial contaminants may destroy or modify Notifier's rhLF. Stability should not only be tested in real time but also under accelerated conditions. Stability analyses should include assays that detect protein modification such SDS PAGE, bioassays, reversed phase HPLC and ELISA. Without proper stability studies, the integrity of Notifier's marketed product cannot be assured.

Reproducibility of lactoferrin source and production

Because analytical methods are not yet sophisticated enough to fully characterize complex proteins like lactoferrin, there exists the need to show reproducibility and stability of the recombinant production system to ensure quality control. Notifier presents virtually no data on the reproducibility of its rhLF production in rice, and the data that are given raise serious doubts about Notifier's ability to control production. For example, there is more than 20% variation in lactoferrin content in subsequent generations of rice plants, a more than 30% difference in yields between batches and

more than 400% variation in the level of impurities and contaminants. These variations would not normally be considered acceptable in the production of therapeutic recombinant proteins.

Notifier's production process lacks adequate controls for genetic stability. In conventional recombinant production systems, genetic stability of the host cell is ensured through the use of master and working cell banks. Additionally, manufacturers are required to demonstrate that no genetic drift occurs during production. However, in the case of Notifier's manufacturing system, each crop is used as a source for the next production crop. This is a risky procedure from the point of view of genetic stability and is especially important because, in contrast with cell or microbial culture systems, rice is cultivated under variable conditions that are impossible to fully control or standardize. To ensure constant product quality it is essential that Notifier provide data on the gene sequences and expression profiles of different plants, under different cultivation conditions in various geographic regions, as well as plants cultivated under different herbicides and pest control systems, and yields from rice stored under different conditions.

Notifier does not provide any of this data and its production controls are inadequate with respect to recognized standards for producing recombinant proteins. Without adequate controls, Notifier cannot provide the public assurances regarding the activity, purity or consistency of its rice-produced rhLF. Furthermore, inadequate product characterization could pose safety risks by failing to identify and quantify harmful impurities or degradation products known to be common with recombinant proteins.

INDIANA UNIVERSITY

DEPARTMENT OF BIOLOGY

November 5, 2005

Recombinant Human Lactoferrin: Unanswered Safety Questions

Our body employs lactoferrin (Lf) as an iron scavenger and antimicrobial agent. The concentration of Lf is maintained at a very low level except when its activity is required. For instance, a normal level in serum is 0.005 μM, going up in septicemia to 2.5 μM. In normal vaginal fluid it is 0.1 μM, going up in genital tract infections to 40 μM. Lactoferrin is not a nutrient.

In saliva, the concentration normally is about 0.1 μM. When employed for specific medical conditions, orally administered Lf products can be used at substantially higher concentrations with appropriate clinical oversight, provided that awareness of the following risks is maintained.

1) Some persons have latent or overt infections due to pathogenic microbes that express receptors to Lf so as to acquire growth-essential iron. Among such examples are Helicobacter pylori, a bacterial cause of gastric ulcers and possibly gastric cancer; various Neisseriaceae such as bronchitis-causing strains of Hemophilus influenzae; and pathogenic protozoa such as Leishmania, Toxoplasma and Trichomonas.

2) Some persons, if they produce a polymorphic variant of Lf, might regard the protein as a foreign antigen. Moreover, antibodies to Lf have been detected in persons with such autoimmune disorders as lupus, theumatoid arthritis, type 1 diabetes, and primary sclerosing cholangitis.

3) Unfortunately, transgenic rice grains expressing rhLf have a twofold increase in iron content. Some persons can sequester the additional burden in their iron storage tissues. Others have little or no excess capacity to do so. The latter include persons with hemochromatosis, sickle cell anemia, myelodysplastic syndrome, or other iron loading disorders.

(continued on p. 2)

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Recommendation:

Recombinant human lactoferrin, a powerful pharmaceutical product, should not be distributed to general, non-selected populations in the absence of rigorous tests for safety. These tests should ascertain that use of the product will not result in an unacceptable rate of toxicities including overgrowth of specific pathogenic microbes, development of allergic hypersensitivities, or increase in iron-associated disease manifestations.

Inasmuch as the various recombinant products differ in glycosylation, each should be individually tested for safety.

E.D. Weinberg, PhD
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Reference: Weinberg, E.D. (2003) The therapeutic potential of lactoferrin. Expert Opinion on Investigational Drugs 12:841-851.

INDIANA UNIVERSITY

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BIOLOGY

November 5, 2005

Dr. Atul Varadhachary
President & Chief Operating Officer
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Dear Dr. Varadhachary,

This memo is to authorize my co-signature on your letter to CFSAN on safety concerns raised by recombinant human lactoferrin produced in rice.

Eugene D. Weinberg, PhD

Eugene D. Weinberg, PhD
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November 9, 2005

Laura M. Tarantino, Ph.D. (HFS-200)
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Center for Food Safety and Applied Nutrition
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Re: Safety Concerns Raised by Recombinant Human Lactoferrin from Rice; GRN No. 000162 Submitted by Ventria Bioscience

Dear Dr. Tarantino:

After participating in the scientific assessment of GRN No. 000162, evaluating safety issues pertaining to the use of rice-produced rhLF as an ingredient in functional foods, I am writing to independently express my concerns on this matter.

As a scientist, I have been involved in evaluating the biosimilarity of recombinant proteins and have both published and consulted on this subject. Advances in technology have brought a host of complex proteins within the range of broad scientific study and large-scale production. This raises exciting new possibilities for the use of complex proteins in the treatment of disease. But it also brings new risks and potential for inappropriate or unsafe use.

Because of their complexity, and the complexity of the living systems use to produce them, recombinant proteins may be inherently ***dissimilar*** to their native analogues in many ways that can profoundly effect their biologic activity and safety. Such dissimilarities can include glycosylation, the inclusion of protein variants, and impurities like host-cell protein and endotoxin. These differences are impossible to completely eliminate from recombinant products, given the current state-of-the-art, and can have a large impact on protein function as well as safety. The differences are increasingly associated with serious immune recognition problems including the generation of antibodies with potential neutralization of endogenous protein.

Given these realities, and the potent biologic effects of rhLF as demonstrated in recent clinical trials in non-small cell lung cancer, it is concerning that rhLF is being considered for use in food by extrapolating tests performed with different products and without extensive safety testing of the product itself. It is also troubling that the scientific case made to support a GRAS designation is fraught with contradictions and inconsistencies.

The following table contains a summary of Ventria's conclusions about rice-produced rhLF, as well as my brief comments based on a review of the data presented.

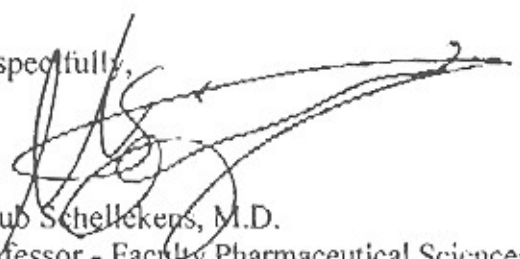
Ventria Conclusions	Scientific Comment
The proteins [rice-produced rhLF and native human lactoferrin] are biochemically equivalent.	This statement is false. It implies that human lactoferrin is a single protein existing in only one form. In reality it is part of a polymorph system with different alleles. Ventria's rhLF is derived form a gene sequence without a clear understanding of the gene's natural occurrence. Moreover, Ventria has submitted data indicating divergences of its product with the amino acid sequence of the natural protein. No data has been presented on the genetic stability of the host rice plants, which is vital to assuring that additional changes to the amino acid sequence do not take place. In addition, there are substantial and important differences in glycosylation that carry known health risks.
The gene source is non-allergenic.	This is irrelevant. The allergic potential of the gene source is not relevant for a risk analysis. What matters is the allergenic potential of the product.
The proteins are not allergens.	This is an unsubstantiated claim. Native LF has been implicated in milk allergy. Rice produced rhLF has characteristics of known allergens, including carbohydrate structures know to cause an IgE response in humans. It remains unknown whether rice-produced rhLF is allergenic. This can only be determined in large clinical trials of prolonged exposure to the product.
The risk of allergic sensitization to lactoferrin is remote.	This statement is false. Risk is frequency times the consequences. Even if the frequency is shown to be low in robust clinical trials, the consequences of allergy are too serious to categorize the risk as remote.
Residual rice protein will not likely trigger an allergic response.	This is an unsubstantiated claim. The level of residual rice protein in the product is high. The rice used to produce rhLF has not been marketed in the U.S., so the allergenic level in the U.S. is unknown. Most important, the protein make-up of the plant is changed by the introduction of the lactoferrin gene. At least one other gene is activated and a number of genes are down regulated. Again, the only way to substantiate the claim is through large human clinical trials.
For the rare individual with true rice allergy, the products will be labeled as recombinant human lactoferrin from rice.	Although these individuals should avoid the product, the allergenic potential in others cannot be excluded by the modifications in the rice. This is of greater concern to the safety of the general public, who have never before been exposed to rhLF from any source, much less from rice.
Recombinant lactoferrin from rice can be regarded as safe for pollen-allergic patients with anti-CCD IgE.	This is an unsubstantiated claim. This can never be concluded from *in vitro* studies with anti-sera. Only challenge studies in pollen-allergic patients will show the safety of the product in allergy patients.

There is no evidence of immunologic cross reactivity with other iron-binding, transferrin-like proteins from humans or other species, some of which may be related to allergic sensitization.	This is an unsubstantiated claim. Such a statement can only be based on experiments that use anti-sera raised from the actual rhLF product and cannot rely on literature data. However, even such experiments would only show a lack of antigenicity, which has a poor correlation with immunogenicity, a potentially more serious health risk.
Based on histamine release, there is no evidence that the biological IgE binding by sera with pre-existing IgE antibodies to plant N-glycan CCD structures, would result in a clinically relevant reaction in pollen allergic individuals.	This is an unsubstantiated claim. Looking at the histamine release with a limited number of anti-N-glycan sera (3 samples) is insufficient to make any credible scientific determination and is no substitute for clinical trials in large patient populations for a prolonged period of time.
It is highly unlikely that ingestion of human lactoferrin as a food supplement, even by individuals with an ongoing autoimmune disease, would have any adverse effect.	This is an unsubstantiated claim. Anti-lactoferrin antibodies are implicated in a number of serious autoimmune diseases. It is highly likely that rice-produced rhLF is immunogenic because it is derived from a gene variant not shared by all individuals. In addition the amino acid sequence of the rhLF seems to be modified and contains foreign sugar residues from rice. To establish safety in this aspect, immunogenicity challenge studies for at least a year in different human populations are necessary.
Using the data generated for this application and other generally available and accepted scientific data, information, methods and principals, there is a reasonable certainty that rhLF from rice will be safe under the intended conditions of use in functional and medical foods.	This statement is false. Only data generated with the product to be marketed are relevant. There are several indications that the product will pose serious risks to public health.

The preceding comments on Ventria's GRAS panel conclusions are dealt with more extensively in the Scientific Assessment accompanying this letter. They serve to illustrate the need for thorough clinical evaluation before coming to any conclusion regarding the safety of rice-produced rhLF in foods. This type of research has not been conducted to date, and a GRAS designation of this product is, in my opinion, inappropriate at this time.

Please do not hesitate to contact me if I may be of further assistance.

Respectfully,



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Attachment 4

Ventria Has Publicly Described Its Intent as the Production of Pharmaceutical Lactoferrin to Treat Human Disease

Timeline of Published Statements by Ventria

Present

In a glossary published on Ventria's corporate website, the company defines its own terms for its products:

***"Biopharmaceuticals*:**

Output Traits: In agricultural biotechnology, input traits are traits that improve the agronomic performance of the plant (i.e., RoundUp Ready(R) Corn). Output traits are traits that change the way the plant is used. In this case, ***Ventria's output traits are biopharmaceuticals***.

Pharmaceutical: Of or pertaining to the knowledge or art of pharmacy, or to the art of preparing ***medicines*** according to the rules or formulas of pharmacy; as, ***pharmaceutical preparations***. ["Biopharmaceuticals" are pharmaceuticals produced in biologic systems.]

Plant-made Pharmaceuticals: The art of preparing ***medicines*** according to the rules or formulas of pharmacy through the use of plants.

Therapeutic Proteins: A protein, of or pertaining to the healing art; concerned in discovering and applying ***remedies for diseases; curative*."**

-- Ventria Bioscience's Website

Oct 19, 2005

A Ventria abstract presented at the 7th International Conference on Lactoferrin makes clear references to Ventria's intended use of rhLF to treat disease:

"Supported by the studies on lactoferrin as an anti-microbial, anti-infammatory and immunomodulatory protein, ***the use of Lactiva [brand name of Ventria's rice-produced rhLF] in gastrointestinal disease provides ideal applications***." The abstract further suggests use of rhLF to treat "children with ***mild to moderate inflammatory bowel disease (IBD)***."

-- Ventria Abstract at the 7th International Conference on Lactoferrin, Honolulu, Hawaii, October 19, 2005

Jun 29, 2005 In testimony before the United States House of Representatives Subcommittee on Rural Enterprises, Agriculture, and Technology, Ventria stated that the company is engaged in developing plant-made pharmaceuticals and biologics intended as medicines: "Ventria Bioscience is a plant-made ***pharmaceutical*** company that utilizes rice and barley as a factory to produce biologic products," and "Ventria believes this technology will lead to more affordable ***medicines*** for a much broader ***patient population*** than what is possible with ***conventional biopharmaceutical production technology*** today."

Ventria also presented, in this recent testimony, medical claims for its rhLF, branded as Lactiva™, stating, "***Ventria believes it [Lactiva]can improve the recovery rate and reduce the severity or duration of diarrhea***" in children, and Ventria is targeting "***Inflammatory Bowel Disease***" and ***"Chronic Lung Infections caused by Pseudomonas"*** in patients with Cystic Fibrosis. This last statement was made despite the fact that a recently published scientific study has implicated Ventria's own lactoferrin in the induction of antibiotic resistance to Pseudomonas in patients with Cystic Fibrosis [Andres MT, Viejo-Diaz M, Perez F, Fierro JF., Antibiotic tolerance induced by lactoferrin in clinical Pseudomonas aeruginosa isolates from cystic fibrosis patients. *Antimicrob Agents Chemother*. 2005 Apr;49(4):1613-6.]

-- From Ventria's Testimony Before The Subcommittee on Rural Enterprises, Agriculture, and Technology, United States House of Representatives, June 29, 2005

Nov 18, 2004 Recent Ventria press release describes intent to produce proteins for pharmaceutical use: "Ventria is a ***biopharmaceutical*** company that has developed a novel production system called ExpressTec that uses self-pollinating plants, rice and barley, as factories to produce ***therapeutic proteins*** and peptides."

-- Ventria Press Release, November 18, 2004

May 27, 2004 Ventria asserts that the company's objective for lactoferrin is an "orally administered ***biopharmaceutical***" (drug) that will be used to treat human disease including bacterial and viral infections, anemia, and diarrhea.

-- Interview with* Rehydrate.org*, May 27, 2004

Apr 16, 2004 Ventria asserts that lactoferrin and lysozyme are intended to treat diseases such as anemia and diarrhea and states: "Ventria sees itself as a biotechnology company hard at work on ***medical*** products that could ***save lives***."

-- "Bioengineered rice takes center of debate over using food crops to grow drugs", **San Jose Mercury News*, April 16, 2004***

Apr 8, 2004 In a leading scientific periodical, Ventria's lactoferrin and lysozyme are described as "drugs" with Ventria's CEO making the unsubstantiated claim that "***Ventria's products have the potential to save the lives of 2 million children a year***."

-- "California OKs GM Pharm Crops" **The Scientist*, April 8, 2004***

Feb 1, 2004 According to *The Independent*, Ventria claims that its plants "will become 'factories' that manufacture ***therapeutic proteins*** to combat ***life-threatening illnesses,***" and that "plants improved through the use of biotechnology" can produce them "***for innovative treatments for diseases such as cancer, HIV, heart disease, diabetes, Alzheimer's disease, kidney disease, Crohn's disease, cystic fibrosis and many others***".

-- "GM Rice To Be Grown For Medicine" **The Independent, *February 1, 2004***

Jan 27, 2004 In an article in the *Sacramento Bee*, Ventria states that the company intends to sell the rice-derived lysozyme and lactoferrin for use in oral rehydration products to ***treat severe diarrhea***. Ventria further states that 65 acres of Ventria rice could generate 1,400 pounds of lactoferrin, enough to ***treat*** at least 650,000 sick children. The same acreage of lysozyme rice would yield enough protein to ***treat*** 6.5 million ***patients***. Notwithstanding Ventria's ubiquitous characterization of lactoferrin as a pharmaceutical, the article infers that Ventria seeks to avoid regulation as such: "Besides state and USDA hurdles, pharmaceuticals also are overseen by the U.S. Food and Drug Administration. But Ventria is categorizing its rice as 'medical food', which does not require FDA review."

-- "A new field of rice", **Sacramento Bee, *January 27, 2004***

Jan 25, 2004 In an article in the *Sacramento Bee*, Ventria claims that its lactoferrin and lysozyme "would be the first genetically engineered plant-produced ***pharmaceuticals*** to reach the market," and that Ventria's rice is intended to *"**treat severe diarrhea**"* and is *"**not intended as food**"*.

-- "Biotech company cultivates new field",* Sacramento Bee, *January 25, 2004

Jun 17, 2003 Ventria makes presentation on plant-made ***pharmaceuticals*** to the USDA in which its principal products, recombinant human lactoferrin and lysozyme, are characterized as ***pharmaceuticals*** with the intent of ***treating diseases*** such as ***acute respiratory infections and diarrhea***.

-- USDA Presentation, June 17, 2003

Oct 24, 2002 In a company press release, Ventria touts the company's technology for producing pharmaceutical drugs in plants: "Ventria's ExpressTec(TM) system is an ideal solution for the production of plant-made ***pharmaceuticals*** and industrials because it utilizes self-pollinating plants to produce ***biopharmaceuticals***. ... The use of plant systems to produce ***biopharmaceutical proteins*** and peptides is an important technology in the fight against ***human disease***."

-- Ventria Press Release, October 24, 2002

Oct 1, 2002 Ventria announces the launch of ExpressTec(TM), its proprietary technology that enables the high level expression of ***therapeutic proteins*** in rice, wheat and barley. "ExpressTec(TM) is a major breakthrough for Plant-made ***Pharmaceuticals***."

-- Ventria Press Release, October 1, 2002

Jul 25, 2001 Applied Phytologics (a.k.a. Ventria Bioscience) applies to APHIS for permit #01-206-01R to grow GMO rice producing rhLF and lysozyme in Hawaii. In its application, the company declares the intended purpose of the GMO rice to be the production of ***Novel Pharmaceutical Proteins***.

-- Source: ISB Environmental Releases Database of the USDA Animal and Plant Health Inspection Service (APHIS)

Jan 29, 2001 Applied Phytologics (a.k.a. Ventria Bioscience) applies to APHIS for permit #01-029-02R to grow 100 acres of GMO rice producing rhLF and lysozyme in California. In its application, the company declares the intended purpose of the GMO rice to be the production of ***Novel Pharmaceutical Proteins***.

-- Source: ISB Environmental Releases Database of the USDA Animal and Plant Health Inspection Service (APHIS)

Jun 1, 2000 In a company press release, Ventria Bioscience states that it is a developer of recombinant proteins that address a variety of human health issues, including ***Infectious Disease, Cystic Fibrosis, and Emphysema***. Ventria states that its corporate mission is to develop products that enhance and ***save lives***.

-- Ventria Press Release, June 1, 2000

DEPARTMENT OF HEALTH & HUMAN SERVICES Public Health Service

Food and Drug Administration
College Park, MD 20740

January 5, 2005

Delia R. Bethell, Ph.D.
Ventria Biosciences
4110 N. Freeway Blvd.
Sacramento, CA 95834

Re: GRAS Notice No. GRN 000162

Dear Dr. Bethell:

The Food and Drug Administration (FDA) has received the notice, dated December 16, 2004, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS)). FDA received this notice on December 21, 2004, filed it on December 22, 2004, and designated it as GRN No. 000162.

The subject of the notice is lactoferrin produced from rice expressing a synthetic gene encoding human lactoferrin (lactoferrin). The notice informs FDA of the view of Ventria Bioscience that lactoferrin produced from rice is GRAS, through scientific procedures, for use as an ingredient in foods and drinks at levels not to exceed 1000 mg/serving and in medical foods at levels not to exceed 1 mg/mL.

In accordance with proposed 21 CFR 170.36(f), a copy of the information in the notice that conforms to the information described in proposed 21 CFR 170.36(c)(1) is available for public review and copying on the homepage of the Office of Food Additive Safety (on the Internet at http://www.cfsan.fda.gov/~lrd/foodadd.html). If you have any questions about the notice, contact me at 301-436-1226.

Sincerely yours,

Robert I. Merker, Ph.D.
Division of Biotechnology and
GRAS Notice Review
Center for Food Safety
and Applied Nutrition

Hard copy cc: **GRN 000162** (1 copy)
Electronic mail cc: HFS-255(LKahl)
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July 19, 2005

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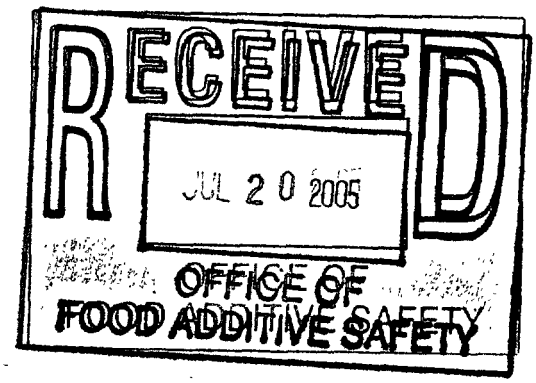


RE: Ventria Bioscience
GRAS Notice No. 000162
Lactoferrin (human) Purified From Rice

Dear Drs. Tarantino, Schneeman, and Martin:

It has just come to our attention that, in order to obtain approval from the USDA to grow its genetically modified rice to produce recombinant human lactoferrin (rhLF), Ventria Bioscience has made public representations implying that FDA/CFSAN scientists and medical experts have "unanimously concluded that lactoferrin (human) derived from rice produced by Ventria is substantially equivalent to and, therefore, as safe as, native human lactoferrin found in

tears, saliva and mother's milk." We think this reference to FDA/CFSAN by Ventria to bolster its arguments to obtain a permit from the USDA is improper.

We quote the full text below of the two paragraphs from Ventria's letter to the USDA/APHIS/BRS (document APHIS-2005-0013-0352) that inappropriately use FDA/CFSAN as support for its argument that recombinant human lactoferrin (rhLF) is as safe as native human lactoferrin:

> **"Safety of Lactoferrin (Human) Derived From Rice:**
> Ventria is developing lactoferrin (human) derived from rice for use in medical foods, functional foods and drinks. In December, 2004 Ventria submitted a GRAS (Generally Recognized as Safe) Notice to FDA/CFSAN for lactoferrin (human) derived from rice as GRN 000162. As part of the GRAS process a panel of scientific and medical experts unanimously concluded that lactoferrin (human) derived from rice produced by Ventria is substantially equivalent to and, therefore, as safe as, native human lactoferrin found in tears, saliva and mother's milk. Mr. Barsky fails to acknowledge these facts in his letter."
>
> **"Consumption of Human Lactoferrin:**
> Human lactoferrin has a history of general consumption, contrary to Mr. Barsky's claim. Human lactoferrin is found in tears, saliva, bronchial fluids and mother's milk. Humans consume up to 6 grams of lactoferrin yearly from saliva. In addition, Ventria has conducted human and animal studies with lactoferrin (human) derived from rice. The results of these studies are consistent with the conclusion of safety for oral consumption reached by the GRAS panel of scientific and medical experts. Mr. Barsky incorrectly states the facts relating to the consumption history of both native and recombinant forms of human lactoferrin, including lactoferrin (human) derived from rice."

These paragraphs convey the impression, at least to those not familiar with the GRAS process, that the "panel of scientific and medical experts" referred to is an independent FDA/CFSAN panel rather than advisors and consultants retained by Ventria.

We would also like to respond to Ventria's other arguments made in these two paragraphs.

I. Novel Molecule With Little Prior History Of Human Consumption.

Ventria's first argument is that recombinant human lactoferrin "is substantially equivalent to and, therefore, as safe as" native human lactoferrin. It is clear, however, from both a scientific and a regulatory point of view, that Ventria's recombinant human lactoferrin is a novel and unique chemical entity not previously found in nature.

Agennix, Inc., manufactures recombinant human lactoferrin in Aspergillus under cGMP conditions. When Agennix requested a USAN name for the product, the USAN Council, based on guidance from the FDA/CBER, responded that the USAN name would need to reflect the unique identity of the Agennix recombinant lactoferrin. Specifically, this name was required to include a prefix reflecting the particular amino acid sequence of the recombinant protein and a suffix reflecting the unique glycosylation pattern, which is different from that found in native human lactoferrin. Based on these considerations, USAN assigned Agennix's recombinant human lactoferrin the unique name of "talactoferrin alfa" **(Attachment 1)**. Both of these considerations would also apply to recombinant human lactoferrin produced in rice -- namely, a specific amino acid sequence, and a glycosylation pattern different from that found in native human lactoferrin.

Thus, recombinant human lactoferrin produced from rice is clearly a unique chemical entity, never previously found in nature and with no meaningful history of general human exposure or consumption. Recombinant human lactoferrin has substantial differences related to glycosylation, and the impact of these glycosylation differences on safety in humans has not been established. The structural differences between native human lactoferrin and Ventria's recombinant version of human lactoferrin may also be significant. The sequence produced by Ventria's genetically modified rice has not been disclosed. We wrote to you on February 4, 2005 to discuss the proper regulatory handling of recombinant human lactoferrin.

II. History Of Human Consumption Of Native Human Lactoferrin.

Breast fed human neonates and infants consume native human lactoferrin, which is found at significant concentrations (1 mg/mL) in human milk. We believe that lactoferrin plays an important and beneficial physiological role in this situation. The neonatal immune system is underdeveloped [1] and lactoferrin probably plays an important role in strengthening the immune status of the infant through its potent immunomodulatory effects, particularly its ability to induce an IL-18 mediated Th1 shift.[2,3]

Since the human exposure through breast milk consumption is limited in duration and found only in a specific subpopulation that is not the focus of the GRAS notification, Ventria argues that native human lactoferrin has a broader history of human consumption because of its presence in trace amounts in human saliva. Ventria asserts that humans consume up to 6 grams of lactoferrin yearly from saliva. This comparison is irrelevant since Ventria's recombinant human lactoferrin is a distinct and unique molecule and any safety experience with native human lactoferrin is not directly transferable to any form of recombinant human lactoferrin. The level of exposure cited by Ventria falls far below the likely usage of this molecule if the FDA were to raise no questions about Ventria's GRAS notification. Agennix currently has human cancer trials underway with recombinant human lactoferrin under an IND where the patients receive doses ranging from 3 grams to 9 grams per DAY, which is far in excess of the "up to" 6 grams

per YEAR cited by Ventria. It is highly likely that dietary supplements containing recombinant human lactoferrin will be consumed in the same grams per day range. In fact, Ventria's GRAS filing states that one of the intended uses is as an ingredient in foods and beverages at levels up to a gram per serving.

Ventria's recombinant human lactoferrin would be administered in combination with contaminants including proteins and other molecules derived from the host cell -- a very different melieu from native human lactoferrin found in saliva -- which could result in an altered physiological or pharmacological effect. Finally, the mode of consumption of recombinant human lactoferrin -- where gram quantities are administered as a bolus -- is fundamentally different from the natural situation where microgram quantities of native human lactoferrin are swallowed over 24 hours on a continuous basis.

III. Concerns About The Safety Of Exogenous LF From The Scientific Community.

Lastly, Ventria argues that it has conducted human and animal studies with its recombinant human lactoferrin satisfactorily demonstrating its safety. Section 170.30(b) of the FDA regulations requires that GRAS determinations be based on publicly available / published safety studies and a clear consensus regarding safety. We are not aware of any such published studies with Ventria's recombinant human lactoferrin that could provide adequate proof of safety, and there is clearly a lack of consensus on the safety of Ventria's recombinant human lactoferrin.

Over the years, the scientific community has expressed concerns about potential safety problems from exogenously administered lactoferrin. Lactoferrin is a potent agent that has a number of important biological properties that raise safety concerns.

- Lactoferrin binds iron with a high avidity across a broad range of pH concentrations.[4] Depending on the context, this can result in lactoferrin functioning as either an iron-chelating agent or an iron-delivery mechanism. In the past, the FDA and university ethics committees have expressed concern that lactoferrin's iron chelation might induce anemia, particularly in vulnerable populations such as pregnant or lactating women. Perhaps of greater concern is the possibility that pathogens might capitalize on iron delivered by lactoferrin. Microbial colonies tend to be iron constrained, and access to a source of iron can induce infectious flare-up. In fact, a variety of bacteria have evolved high affinity lactoferrin receptors that enable them to extract the iron bound to lactoferrin.[5] The role of iron as a growth-regulating factor applies more broadly beyond micro-organisms. The growth of tumors is also known to be iron regulated, and increased dietary iron has been shown to promote colon tumors in mice.[6,7] Tumor cells are also known to over-express receptors that bind lactoferrin with a high affinity **(Attachment 2)**. Thus, there is a risk that precancerous or early stage GI tumors could also access lactoferrin containing iron to accelerate their growth and metastasis.

- Another of lactoferrin's biological properties is its ability to interact with microbial membranes, resulting in a variety of effects including depolarization. A recent publication using the recombinant human lactoferrin produced by Ventria states that exposure to recombinant human lactoferrin induces antibiotic tolerance in Pseudomonas aeruginosa, an important pathogen responsible for numerous hospital infections **(Attachment 3)**.

- Microorganisms have also evolved other ways to capitalize on the trace amounts of lactoferrin to which they are naturally exposed. For example, it was recently described that exposure of pathogenic streptococci to lactoferrin results in the induction of the streptococcal pyrogenic exotoxin A.[8]

- Lactoferrin also has an allergenic potential, and several epitopes found in the protein are known allergenic sequences.[9] Thus, the broad human exposure in an uncontrolled setting that may follow a GRAS determination might result in serious adverse events from allergic reactions to lactoferrin as well as increased sensitivity to other molecules sharing these allergic epitopes. These effects may be further amplified by the low level allergens found normally in rice itself.[10]

- It has become clear that oral lactoferrin also exerts a potent immunostimulatory effect including the production of key cytokines in the gut and in systemic circulation, an increase in circulating CD4+ and CD8+ cells and an increase in NK cell activity.[11] These biological effects are important for pharmacological applications of lactoferrin (including the treatment of cancer). The long-term effect of chronic immunostimulation, including the possible induction of autoimmune diseases, is not known. There have been several recent examples of serious adverse events resulting from long-term administration of immunomodulatory agents, including most recently Tysabri. Risks of this nature may be acceptable when dosing is limited to patients under the supervision of a physician, especially those with serious diseases like cancer, though that determination will ultimately be made by the FDA/CDER. But clearly a determination at this point that long term unsupervised dosing with human lactoferrin is safe, is premature.

Thus, a broad set of published work by scientific experts in the field articulate the very real concerns about safety and, at a minimum, clearly demonstrate a lack of scientific consensus regarding the safety of recombinant human lactoferrin. These genuine safety concerns, which have been published in reputable scientific journals, are best addressed by evaluation of the molecule in animal studies and long term clinical trials.

IV. Clinical Safety Experience With Recombinant Human Lactoferrin.

Agennix has been conducting clinical trials with oral rhLF under INDs filed with the FDA since 1996, and has accumulated an extensive safety database. Agennix's primary focus for oral rhLF is for the treatment of patients with cancer. Since cancer is a major and life threatening disease, even relatively toxic chemotherapeutic agents have been approved for the treatment of cancer, following demonstration of efficacy.

Agennix's experience suggests that rhLF may be effective in treating patients with cancer. In a double-blind, placebo-controlled Phase II clinical trial in first line treatment of patients with advanced non-small cell lung cancer, the addition of oral rhLF to standard chemotherapy substantially improved response rates and other tumor related endpoints relative to the chemotherapy alone. Assuming significant cancer efficacy is confirmed in Phase III clinical trials, the safety profile of rhLF may be acceptable for this patient population, although that determination would need to be made by the FDA/CDER following evaluation of the Phase III clinical data.

Agennix has been monitoring and reporting on the safety and efficacy of oral rhLF through its clinical development program. A partial list of adverse events reported for patients enrolled in rhLF trials is provided in Table 1.

Table 1. CTC Grade 1-3 Adverse Events Reported in rhLF Clinical Trials

Gastro-Urinary	Hematuria
	Nocturia
	Dysuria
	Proteinuria
	Urinary Hesitancy
Digestive System	Nausea
	Vomiting
	Diarrhea
	Constipation
	Dyspepsia
	Anorexia
	Gastritis
	Gastroenteritis
	Stool Abnormality
	Flatulence
	Abdominal Cramps
	Heartburn

Body as a Whole	Abdominal Pain
	Headache
	Fever
	Hot Flashes
	Fatigue
	Malaise
Respiratory	Cough
	Respiratory Distress
	Rhinitis
	Sleep Apnea
	Hiccup
Skin and Appendages	Rash
	Pruritus
Special Senses	Taste Perversion
Hemic and Lymphatic	Anemia
	Hypochromatosis
	Hyperuricemia
	Hypercalcemia
	Hyperkalemia
	Elevated Creatinine
	Edema
Nervous System	Insomnia
Cardiovascular	Hypotension
	Sinus Tachycardia

We do not believe that all of these adverse events were caused by recombinant human lactoferrin. The final determination of recombinant human lactoferrin's clinical safety will be made based on our placebo controlled Phase III clinical trials, which will be the basis for a possible marketing approval by FDA/CDER.

Agennix has spent over $70 million developing recombinant human lactoferrin, and hopes to begin Phase III trials this year in non-small cell lung cancer and diabetic neuropathic ulcers. Over 440 people have been treated to date with Agennix's oral recombinant human lactoferrin (at centers including M.D. Anderson, Stanford, University of Chicago, UCLA, Cleveland Clinic, and Baylor) and the Company is confident that it and its collaborators have generated and published far more data on recombinant human lactoferrin than Ventria. However, even Agennix would not claim that it has established the level of safety required for a GRAS determination of this biologically active recombinant protein, and Agennix has not yet been permitted by the FDA/CDER to market recombinant human lactoferrin.

V. Recombinant Human Lactoferrin Is A Drug, Has Been Regulated As A Drug, And Should Continue To Be Regulated As A Drug.

Aside from the lack of published data proving the safety of Ventria's recombinant human lactoferrin, and the fact that this compound has no meaningful history of human exposure or consumption, there is an important policy issue within the FDA that should be addressed. Recombinant human lactoferrin is a drug and has been in clinical development as such by Agennix since 1996. Ventria claims (on its website, in published presentations and in press releases) that its recombinant human lactoferrin is intended to be used to treat acute diarrhea, acute respiratory infections, topical infections, fungal infections, anemia, and inflammation.

In testimony on June 29, 2005, before the United States House of Representatives Subcommittee on Rural Enterprises, Agriculture, and Technology, Ventria stated that the company is engaged in developing plant-made pharmaceuticals and biologics intended as medicines: "Ventria Bioscience is a plant-made ***pharmaceutical*** company that utilizes rice and barley as a factory to produce biologic products," and "Ventria believes this technology will lead to more affordable ***medicines*** for a much broader ***patient population*** than what is possible with ***conventional biopharmaceutical production technology*** today" **(Appendix 4)**.

The company also presented medical claims for Ventria's recombinant human lactoferrin, branded as Lactiva™, stating that "***Ventria believes it [Lactiva] can improve the recovery rate and reduce the severity or duration of diarrhea***" in children with this disease, and that Ventria is targeting "Inflammatory Bowel Disease" and "Chronic Lung Infections caused by Pseudomonas" in patients with Cystic Fibrosis **(Attachment 4)**. This last statement was made despite the fact that a recently published scientific study has implicated Ventria's own lactoferrin in the induction of antibiotic resistance to Pseudomonas in patients with Cystic Fibrosis **(Attachment 3)**. Ventria has made several other public statements characterizing their activities as pharmaceutical drug development **(Attachment 5).**

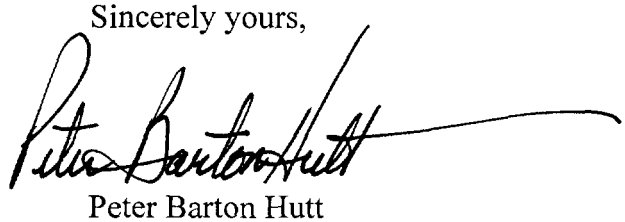
Not only is Ventria talking about the drug properties of recombinant human lactoferrin before Congress, on its website and in the press, Ventria has even published an article on recombinant human lactoferrin's efficacy as an antibiotic comparable to approved antibiotic drugs **(Attachment 6)**. It is obvious that recombinant human lactoferrin's effects as a drug are the reasons Ventria is seeking to produce it and will be the reason people purchase it, and that its use in dietary supplements would be a subterfuge.

Agennix has been following all of the FDA guidelines and requirements for drug development for many years at a cost to date of over $70 million. CFSAN should not be a back door to approval of drugs already in development at CDER. Not only would that unfairly disadvantage those diligently following appropriate drug development procedures, and disincentivise drug development for indications like graft vs. host disease (for which Agennix has been granted orphan drug status) and diabetic foot ulcers (for which Agennix has been

awarded Fast Track status), it would also raise the question of whether there should be two wholly inconsistent safety standards and regulatory pathways for drug approval at the FDA.

Please do not hesitate to contact us if we can provide any additional information. We can also arrange a meeting with Agennix if that would be helpful.

Sincerely yours,



Peter Barton Hutt

Attachments

References

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[11] Varadhachary A, "Oral lactoferrin inhibits growth of established tumors and potentiates conventional chemotherapy." *Int J Cancer*. 2004 Sep 1;111(3):398-403

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Sophia V. Fuerst
Program Director
USAN Council

April 29, 2004

XX 56

Agennix Incorporated
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Attn: Don Martin
Director, Regulatory Affairs
and Quality Assurance

Dear Mr. Martin

It is my pleasure to inform you that the USAN Council adopted **talactoferrin alfa** as the United States Adopted Name for LF09, a recombinant human lactoferrin intended for use as an anti-infective (antimicrobial and antiviral), an anti-inflammatory, and an antineoplastic.

Please review this information for accuracy, initial, and return the statement to me within 60 days of the date listed above. Regardless, after July 1, 2004, the information will be scheduled for posting in the "What's New" section of the USAN web site (www.ama-assn.org/go/usan). At the same time, the information on **talactoferrin alfa** will be submitted to the United States Pharmacopeial Convention, Inc., for publication in the *USP Dictionary of USAN and International Nonproprietary Names*.

You may mail, fax, or e-mail any changes regarding the publication of **talactoferrin alfa** to me at any time before July 1, 2004.

Sincerely,

Sophia V. Fuerst
Program Director and
Secretary, USAN Council

enclosure N04/54

Labelling of human recombinant lactoferrin with technetium-99m and *in vitro* uptake in human tumour cell lines

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Abstract

Lactoferrin (Lf) is an iron-binding protein. The recombinant form of human lactoferrin (rhLf) holds considerable therapeutic potential, especially in the treatment of cancer and gastro-intestinal disorders. The potential for rhLf to act as a cancer radioimaging agent when conjugated with a suitable radionuclide, technetium-99m (^{99m}Tc) is discussed herein through the investigation of the uptake of the ^{99m}Tc-labelled rhLf in a variety of human tumour cell lines.

A method for efficiently labelling rhLf with the medical-imaging isotope ^{99m}Tc has been developed, resulting in a high labelling efficiency (85%) and stability (75%). ^{99m}Tc-labelled rhLf has been incubated with a range of human tumour cell lines from various tissues: A549 (lung), COLO205 (colo-rectal), RT112 (bladder), MCF7 (breast), PC3 (prostate), OVCAR-3 (ovary) and SK-N-SH (brain); and PNT2-C2, a non-tumorigenic cell line from prostate. The uptake of ^{99m}Tc-labelled rhLf has been measured into each cell line at various time points up to 2 h by measuring the incorporated radioactivity using a gamma counter.

RhLf was found to be rapidly taken up into all the cell lines tested and that the cell uptake in the tumorigenic prostate PC-3 cells was 3-fold greater than the uptake in non-tumorigenic prostate PNT2-C2 cells. The uptake occurred quickly in the first 15 min after which it formed a plateau and the maximum uptake was observed after 15-30 min. The cell internalisation of

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the ^{99m}Tc-rhLf was determined and it was found that 25-30% of the activity was internalised after 2 h.

Introduction

The transferrins are a small group of iron binding proteins containing serum transferrin (sTf), lactoferrin (Lf), ovotransferrin (oTf) and melanotransferrin (mTf) [1]. These proteins consist of a single polypeptide chain which folds to form two homologous lobes, C-terminal lobe and N-terminal lobe, each of which contains one iron-binding site. Iron free protein, the apo-form, has an open structure that closes on binding of iron. Iron binding occurs together with a synergistic anion, usually bicarbonate, and the iron-saturated form is known as the holo-form. We have investigated the potential of serum transferrin (sTf) and lactoferrin (Lf), when coupled to a suitable radionuclide – technetium-99m (^{99m}Tc), to act as tumour imaging agents. ^{99m}Tc provides near perfect qualities for clinical imaging, with a half-life of 6 h and γ-rays emissions of 141 keV [2].

STf has a high affinity for Fe^{3+} and it is responsible for the *in vivo* transport of iron in mammals [3]. Transferrin is taken up into the cell by receptor-mediated endocytosis [4-6]. It has been shown that transferrin receptors are upregulated on the surfaces of tumour cells [7-9] presenting this protein as a candidate for cancer targeting. Previous studies by Smith and Walton [10] have made use of the metal binding affinity of transferrin to label it with ^{99m}Tc. *In vitro* experiments using human breast cancer cells (MCF7) and bladder cancer cells (RT112) they show good potential for ^{99m}Tc-labelled transferrin to act as a cancer-imaging agent [10]. In subsequent *in vivo* experiments Smith *et al.* [11] used Tc-labelled sTf to image 2 mm xenograft tumours in nude mice however the low systemic clearance rate of transferrin of several days resulted in a high background count. The related protein lactoferrin (Lf) possesses several key advantages over sTf for imaging purposes. Importantly, the blood

clearance rates of Lf are of the order of 10 min in rats [12,13] and 90 min in man [14], which is ideal for most imaging procedures, and since human Lf is available commercially in a recombinant form, there is no risk of cross-haematological infection.

Lactoferrin is an 80 kDa glycoprotein that is found in various mammalian secretions such as tears, saliva and seminal fluid and it is also present in high concentration in the granulocytes of neutrophils [15-16]. Lf has a very similar structure to sTf showing a sequence identity of ~60% [1,17]. In common with sTf, Lf has a high affinity for Fe^{3+} but it has been shown to retain iron at a lower pH than sTf. The low level of Lf in the bloodstream (<1 μg ml^{-1}) and high affinity of Lf for iron suggests a role as an iron scavenger rather than iron transport protein. Lf has a variety of functions, reviewed in [18-20] however it is its interaction with cancer cells that provides the interest for this study.

Lactoferrin has been shown to bind to discrete lactoferrin receptors on the surface of cancer cell lines and may exhibit some tumour targeting potential. Human Lf has been shown to bind to cells from a range of tissues such as leukaemic cells [21,22], breast [23,24], mitogen-stimulated peripheral blood lymphocytes [25], lymphoblastic T-cells [26], intestinal epithelial cells [27]. The method of uptake of lactoferrin remains unclear at present. Several groups have investigated the cellular binding sites for Lf and in Jurkat human lymphoblastic T-cells [28,29] and adenocarcinoma HT29-D4 cells [30] and they have found that there are a small number of high affinity binding sites, which are proposed to be an Lf receptor, and a greater number of low affinity binding sites, which are shown to be due to cell surface proteoglycans. In MDA-MB-231 breast cancer cells, containing a high level of proteoglycans, the Lf appears to bind to heparan-sulphate proteoglycans but not chondroitin-sulphate proteoglycans [22]. In intestinal epithelial cells the heparin-binding site on Lf is thought to be important for the internalisation of Lf [20].

Recently, Lf has been shown to bind to the protein nucleolin present on the cell surface [24]. Nucleolin is a 105 kDa protein which has a role in cell proliferation however it also functions as a cell surface receptor, where it acts as a shuttling protein between the cytoplasm and nucleus [31]. Nucleolin is thought to be a marker of endothelial cells in angiogenic blood vessels [32]. Legrand *et al.* [24] show that nucleolin also improved the binding of Lf to heparan-sulphate proteoglycans and that it is involved in the cellular uptake of Lf. Nucleolin-Lf was detected in the cell nucleus suggesting that nucleolin may act as a shuttle for the transport of Lf to the nucleus. Lf has also been found in the nucleus of human leukaemia K562 cells [33].

When investigating binding of lactoferrin to human cells the use of human Lf (hLf) is recommended since human Lf receptors have a much higher affinity for the human protein rather than for bovine Lf (bLf). The amino acid sequences of bLf and hLf are only 68% homologous [34,35] and the biological properties of the two molecules may differ significantly [36]. The availability of commercial supplies of recombinant hLf (rhLf) has facilitated investigations into the therapeutic potential of the protein. RhLf is over-expressed in, and isolated from *Aspergillus niger* var. *awamori*. It is structurally and functionally equivalent to native human lactoferrin in all material respects [37], differing only in the nature of its glycosylation [38] and its *in vitro* and *in vivo* use have been reviewed [39]. RhLf's immunostimulatory and anti-cancer activities have been demonstrated in several models, including animals with established tumours more analogous to patients with cancer [40].

In this paper we have investigated the potential for Tc-labelled rhLf to act as a tumour imaging agent by investigating the uptake of this agent into a range of human tumour cells lines from various tissues: A549 (lung), COLO205 (colo-rectal), RT112 (bladder), MCF7 (breast), PC3 (prostate), OVCAR-3 (ovary) and SK-N-SH (brain); and a non-tumorigenic cell line PNT2-C2 from prostate. The A549, COLO205, MCF7 and OVCAR-3 cell lines were

selected for use by being part of the NCI/NIH cell-screening panel [41]. The RT112 cells and MCF7 cells had been used previously with transferrin [10,11] and were therefore included in the lactoferrin study. We also included the SK-N-SH brain cancer cell line, to investigate the utility of this agent in brain imaging. PNT2-C2 prostate cells were used as a comparison for a non-tumorigenic 'normal' cell lines since this cell line displayed all the characteristics of 'normal' prostate tissue and could be compared with the PC3 prostate cells that are derived from cancerous tissue.

Materials and methods

Materials

Human r-Lf was supplied by Agennix, Inc., Houston, Texas (USA) and was snap frozen in small aliquots at –20 °C. It was used without further purification. Pertechnetate ($^{99m}TcO_4^-$), obtained from a technetium generator, was supplied by the Nuclear Medicine department of the Leeds General Infirmary, Leeds, UK. Other laboratory chemicals were purchased from Sigma-Aldrich (Gillingham, UK) and all solutions were prepared in 1× PBS pH 7.4 (Invitrogen, UK) herein referred to as PBS. Prostate PNT2-C2 cells were kindly supplied by Prof. Norman Maitland, Department of Biology, University of York, UK.

Equipment

Centrifugation steps were carried out using an MSE Centaur II benchtop centrifuge. ^{99m}Tc was quantified using a Packard Cobra-Autogamma model 5002 gamma counter. UV-vis spectophotometry was carried out using a spectrophotometer (Perkin Elmer)

Radiolabelling

rhLf labelling with technetium-99m (^{99m}Tc) was carried out as described previously [42]. Briefly, a 500 μl reaction was prepared containing final concentrations of 1 mg ml^{-1} rhLf (~12.5 μmol dm^{-3}), 10 μmol dm^{-3} thiourea, 0.05 mmol dm^{-3} $SnCl_2$ and including 25 MBq $^{99m}TcO_4^-$. The $SnCl_2$ was added last to commence the labelling. Labelling reactions were incubated at 37 °C for 60 min. After which 0.5 ml PBS was added and the ^{99m}Tc-labelled rhLf was purified from the free ^{99m}Tc using Centricon YM-30 filters according to the instructions supplied with the filters. An aliquot of the filtrate, containing the unbound ^{99m}Tc, and an aliquot of the purified Tc-labelled rhLf were counted on a gamma counter using a window of 90–180 keV. The labelling efficiency was determined by comparing the activity recovered from the filter with the total activity recovered from the filtrate and the filter.

Cell culture

Uptake studies were carried out using the following human tumour cell lines: MCF7 breast, RT112 bladder, A549 lung, COLO 205 colo-rectal, SK-N-SH brain, OVCAR-3 ovary, PC3 and PNT2-C2 prostate cells. The MCF7 and RT112 cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% foetal calf serum, 2 mmol dm^{-3} L-glutamine and 10000 units ml^{-1} of penicillin/streptomycin. A549 cells were maintained in RPMI 1640 Medium, supplemented with 50 mmol dm^{-3} sodium pyruvate, 10% foetal calf serum, 2 mmol dm^{-3} L-glutamine and 10000 units/ml of penicillin/streptomycin. PC3 and PNT2-C2 cells were maintained in Hams F-12 Medium supplemented with 10% foetal calf serum, 2 mmol dm^{-3} L-glutamine and 10000 units ml^{-1} penicillin/streptomycin.

Cells were maintained in 75 cm^2 tissue culture flasks in a 5% CO_2 incubator at 37 °C. Cells were sub-cultured 3-4 d prior to an experiment into 18 25 cm^2 tissue culture flasks typically containing 10^6 cells per flask. The cells were confluent at the time of each experiment.

***In vitro* cell uptake assay**

Typically 18 flasks of cells were used per cell uptake experiment to give 6 timepoints in triplicate. The medium was removed and each flask of cells was washed with 3 5 ml PBS. To each flask of cells was added 4 ml Medium 199, containing 600 kBq Tc-labelled rhLf. The flasks were incubated at 37 °C for 5, 10, 15, 30, 60 or 120 min. Following incubation, the medium was removed and the cells were washed with 5 5 ml of PBS. The cells were removed from each flask by the addition of 1 ml trypsin and incubation for 10 min at 37 °C. The cells were pelleted by centrifugation at 500 g for 10 min. For each sample, the supernatant was removed into a fresh tube and 100 μl 5.5 mol dm^{-3} NaOH was added to the supernatant. The cell pellet was resuspended in 1 ml 500 mmol dm^{-3} NaOH. The cell suspension and supernatant samples were counted using a gamma counter and the results, in cpm, were corrected for decay of the ^{99m}Tc over the course of the experiment. A protein assay was carried out on the supernatant and cell pellet samples. Results are expressed as the total uptake

of the ^{99m}Tc-labelled rhLf by each flask of cells, which is defined as the sum of the radioactivity recovered in the supernatant and cell pellet divided by the total protein content, and the internalised Tc-labelled rhLf, which is defined as the radioactivity in the cell pellet divided by the total protein content.

Protein determination

The supernatant and cell pellet samples were neutralised by the addition of 0.25 ml 2 mol dm^{-3} HCl. The protein content was determined using a bicinchoninic acid kit according to the instructions provided with the kit. Briefly, the 1 mg ml^{-1} BSA protein standard, supplied with the kit, was serially diluted with PBS to give 100 μl of a 1, 0.8, 0.6, 0.4, 0.2 and 0 mg ml^{-1} solution. 100 ml of bicinchoninic acid reagent was activated by the addition of 2 ml of the supplied copper sulphate reagent. 100 μl of each test solution was added to a fresh tube and 2 ml bicinchoninic acid reagent added. The solutions were mixed and left standing at room temperature (20 °C) for 3 h. After incubation, the solutions were transferred to 1.6 ml disposable plastic semi-micro cuvettes (Fisher Life Science) and the A_{562nm} determined by UV-vis spectrophotometry. The protein content of supernatant and cell pellet samples were determined from a protein standard curve plotted from the BSA protein standards.

Results

The labelling of rhLf with ^{99m}Tc was carried out in the presence of 25 MBq of $^{99m}TcO_4^-$ and 25 μmol dm^{-3} $SnCl_2$ and results in a labelling efficiency of 88% (±2.6%) and a stability of labelling of 66% (±4.5%), data not shown. The resulting ^{99m}Tc-labelled lactoferrin was incubated with each of the human tumour cells lines and the resulting cell uptake determined over a time-course of 120 min. Six time points were used of 5, 10, 15, 30, 60 and 120 min and each time point was carried out in triplicate per experiment. Each experiment was completed at least twice to yield a minimum of 6 results per time point.

Figure 1, shows a time course for the uptake of ^{99m}Tc-labelled rhLf into A549 cells. The graph shows that the initial uptake is very rapid in the first 5 min increasing over the first 30 min after which the graph forms a plateau. Use of trypsin to cleave any externally bound rhLf reveals an increase in the internalisation of the ^{99m}Tc-rhLf with increasing time. Cell internalisation in A549 cells accounts for *ca.* 30% of the total uptake. A similar pattern of cell uptake of ^{99m}Tc-labelled rhLf is seen in all of the human cell lines tested, see figures 1-7 in the ESI. In common with the ^{99m}Tc-rhLf uptake observed in the A549 cells, the uptake observed in the PC3, RT112, OVCAR-3, COLO-205 cells is very rapid in the first 5 min increasing over the first 30 min after which a plateau is formed. With the MCF7, PNT2-C2 and SK-H-SN cells the graphs show an initial rapid uptake in the first 15 min followed by a slow increase that still appears to be increasing at 120 min. A comparison of the cell uptake data for ^{99m}Tc-labelled rhLf for each of the cell lines is shown in table 1. The uptake is greatest in RT112 bladder cancer cells, showing an uptake of *ca.* 850 000 cpm/mg protein at 60 and 120 min. The second highest uptake is seen in SK-N-SH brain cells followed by OVCAR3 ovary with the least uptake observed in the PNT2-C2 prostate control cells.

As observed in the cell uptake graphs, figure 1 and figures 1-7 in the ESI, the ^{99m}Tc-labelled rhLf is internalised into all of the cell lines with increasing time. In the first 5 min there is a

rapid internalisation of rhLf after which there is a slow increase in internalisation with time. The amount of rhLf internalised increases even in the cell lines in which the total uptake forms a plateau. Of the total ^{99m}Tc-labelled rhLf that is taken up by the cells, the percentage that is internalised at 60 and 120 min is slightly different for all the cell lines, table 1. After 60 min 20-30% of the total uptake of Tc-labelled rhLf is internalised, which increases to 25-40% after 120 min. In general the cell lines that have a lower total uptake of ^{99m}Tc-labelled rhLf, A549, COLO205, PNT2-C2, MCF7, display a greater percentage internalisation at 60 and 120 min.

Figure 2, shows a comparison of the two cell lines that are from prostate tissue. The PC3 cells are cancerous however the PNT2-C2 do not form cancers and are a good model of normal prostate tissue. The total uptake of ^{99m}Tc-labelled rhLf is around three-fold greater in the PC3 cells than in the PNT2-C2 cells. However at 120 min, the percentage internalised is 39.4% with the PNT2-C2 cells compared with 28.3% using the PC3 cells.

A control experiment was carried out to investigate the uptake of ^{99m}Tc-labelled human serum albumen in PC3 cells, figure 3. The total uptake of *ca.* 10000 cpm/mg protein is 30 times less than the uptake observed with ^{99m}Tc-labelled rhLf.

Discussion

The cell uptake of ^{99m}Tc-labelled rhLf occurs very rapidly in the first 5 min after which it increases steadily forming a plateau after incubation for 15 to 30 min, in a majority of the cell lines. The internalisation of the ^{99m}Tc-labelled rhLf occurs rapidly and the amount of internalised protein increases with increasing time for every cell line. The results show that 20-30% of the ^{99m}Tc-labelled lactoferrin is internalised after 60 min increasing to 25-40% after 120 min, depending on the cell line.

All the cell lines tested are able to bind rhLf and internalise cell bound rhLf showing that rhLf receptors are probably present on a wide range of cells however the total cell uptake was different for each of the cell lines indicating that the presentation of rhLf binding sites on the cell surface is cell line dependent. The cell lines that showed the most efficient rhLf uptake are RT112 (bladder) and SK-H-SN (brain) followed by OVCAR-3 (ovary), PC3 (prostate), COLO205 (colo-rectal), A549 (lung) and MCF7 (breast). If rhLf were to be used in cancer imaging, tumours that contain higher levels of rhLf binding sites would prove easier to image than those with a lower level however it is the difference in receptor expression in the tumour compared with the surrounding normal tissue that is most important for imaging.

We found that the uptake of ^{99m}Tc-labelled rhLf in tumorigenic PC3 prostate cells is three times greater than in non-tumorigenic 'normal' PNT2-C2 cells. PNT2-C2 cells are normal prostate epithelial cells that have been immortalised by infection with SV40 [43] however these cells are not tumorigenic in mice, non-invasive and behave like regular prostate cells [44]. These cells are robust and grow readily in culture and are therefore the best model available for normal prostate tissue. The increased cell uptake that was observed in the tumorigenic PC3 cells is evidence that Lf is preferentially taken up and internalised in cancer cells compared with normal tissue.

The uptake of lactoferrin into cells appears to occur through two routes, high affinity sites that are likely to be a classical lactoferrin receptor or low affinity sites where the uptake is mediated through interaction with proteoglycans [23-25] and related proteins such as nucleolin [24]. On certain cell lines it has been shown that there are fewer of the high affinity sites and many more of the low affinity binding sites. The presentation of these two types of site on the cell surface appears to be dependent on the cell type. We have investigated a range of different cell lines and since there is rapid uptake of the rhLf, this shows that each of the cell lines probably displayed receptors for rhLf however it is not possible to say whether these are high or low affinity sites. The fact that the internalisation of the rhLf is rapid for all of the cell lines suggests the presence of high affinity receptors on the cell surface that are able to internalise quickly the cell bound rhLf. The internalisation of bound Tc-labelled rhLf slowly increases with time suggesting the recycling of rhLf receptor or the presence of low affinity sites that can internalise the rhLf more slowly. Legrand *et al.* [24] show that the protein nucleolin plays a role in the internalisation of lactoferrin through the low affinity proteoglycan binding sites. Therefore the expression of factors such as nucleolin in each of the cells would determine the rate at which lactoferrin can be internalised in each cell line.

In conclusion, we have shown the ^{99m}Tc-labelled rhLf is rapidly taken up into a range of human tumour cell lines and it is readily internalised into all the cell lines. There is a 3-fold increase in uptake over control cells and the uptake is negligible when human serum albumen is used. We conclude that there is a good potential for rhLf to act as a tumour-targeting agent which when labelled with ^{99m}Tc may prove useful for tumour imaging.

Acknowledgements

We thank Vistatec York Ltd for funding and Prof Norman Maitland, University of York for supplying PNT2-C2 prostate cells. RhLF was kindly provided by Agennix, Inc.

Figures

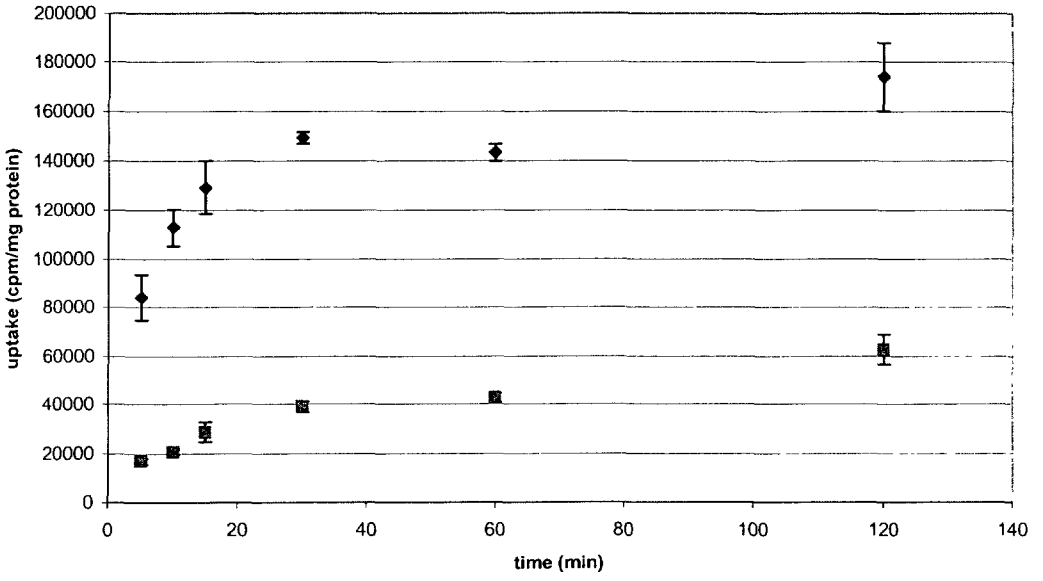

Figure 1. Uptake of ^{99m}Tc-labelled rhLf into A549 human lung cancer cells. (◆) total cell associated uptake; (■) internalised uptake, in cpm/mg protein. The error bars show the standard deviation of at least three points.

Cell Line	Uptake after 60 min (cpm/mg protein)	Uptake after 120 min (cpm/mg protein)	Internalisation of ^{99m}Tc-labelled rhLf after 60 min (%)	Internalisation of ^{99m}Tc-labelled rhLf after 120 min (%)
RT112	880 821 (297 666)	812 764 (238 552)	31.4 (1.5)	34.3 (1.3)
SK-N-SH	458 331 (63 031)	554 460 (107 855)	19.3 (6.1)	23.1 (4.7)
PC3	314 727 (23 250)	299 977 (19 496)	24.2 (2.3)	28.3 (1.9)
OVCAR3	304 159 (35 021)	318 019 (53 675)	22.7 (3.7)	26.5 (6.1)
COLO205	167 188 (23 691)	185 592 (16 334)	36.3 (7.7)	39.9 (1.6)
A549	143 185 (3 400)	173 815 (13 833)	29.9 (2.1)	36.2 (3.5)
MCF7	96 489 (6 071)	122 743 (10 414)	30.2 (4.5)	35.7 (8.6)
PNT2-C2	94 524 (14 642)	111 469 (13 967)	30.2 (3.1)	39.4 (3.4)

Table 1. Summary table showing the uptake of ^{99m}Tc-labelled rhLf into human tumour cells. The figures in brackets are the standard deviation

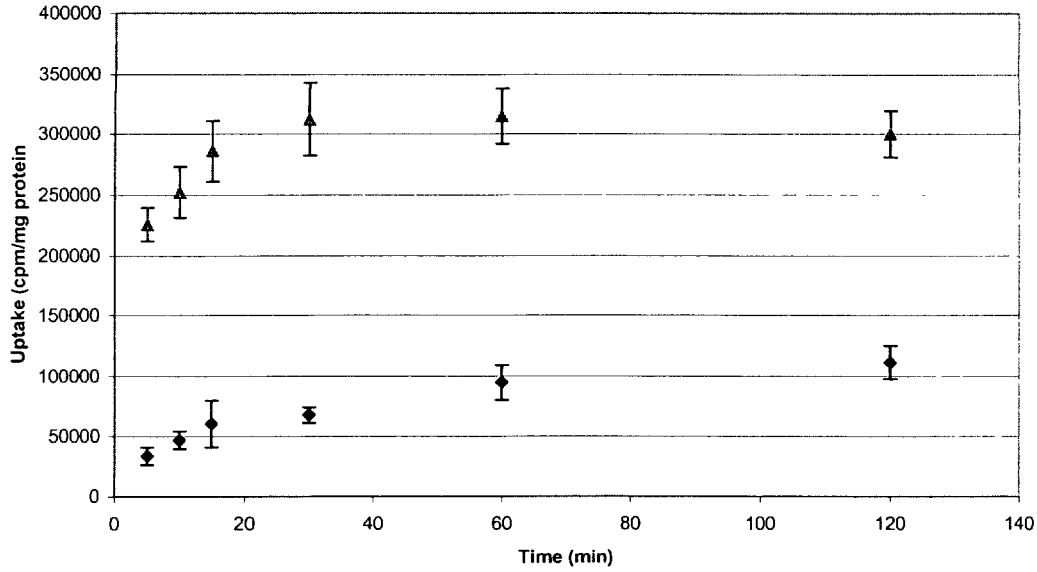

Figure 2. Comparison of the total uptake of ^{99m}Tc-labelled r-Lf in tumorous PC3 cells (▲) and non-tumorous PNT2-C2 cells (◆).

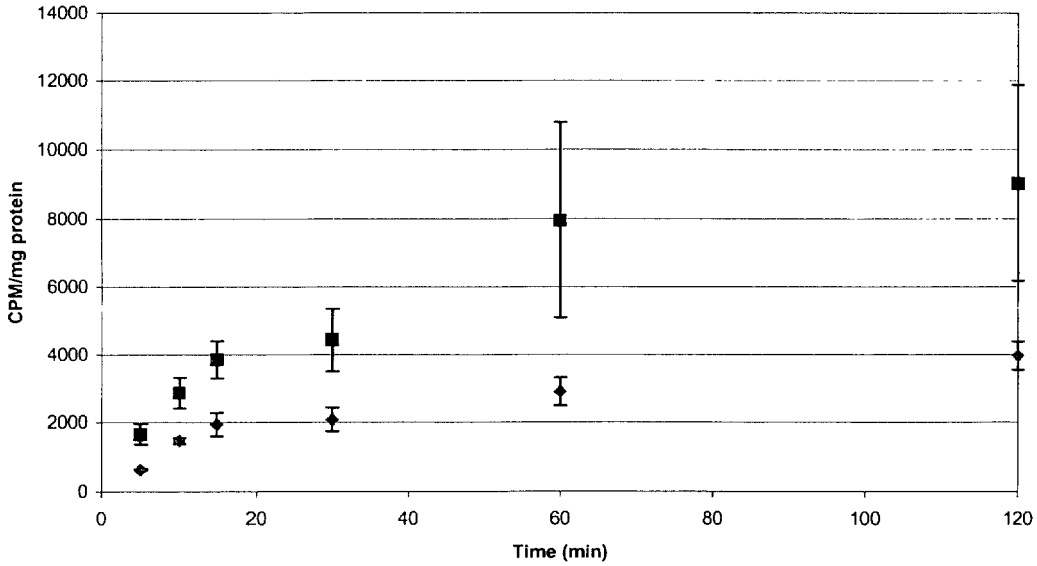

Figure 3. Uptake of Tc-labelled human serum albumen into PC3 cells. (■) total cell associated uptake; (◆) internalised uptake, in cpm/mg protein. The error bars show the standard deviation of three points.

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Electronic Supplementary Information (ESI)

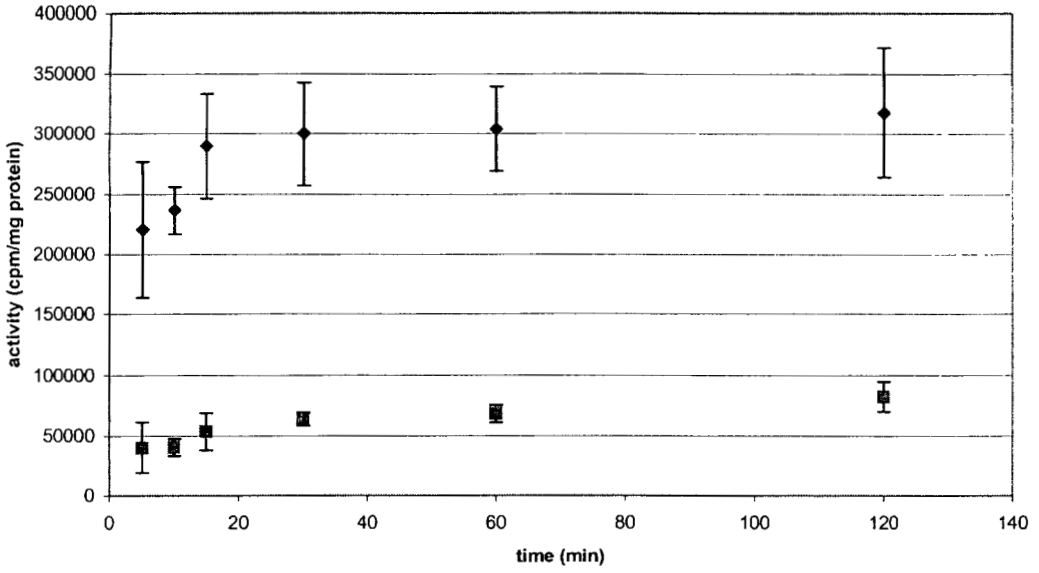

Figure 1. Uptake of ^{99m}Tc-labelled rhLf into OVCAR-3 human ovary cancer cells. (◆) total cell associated uptake; (■) internalised uptake, in cpm/mg protein. The error bars show the standard deviation of at least three points.

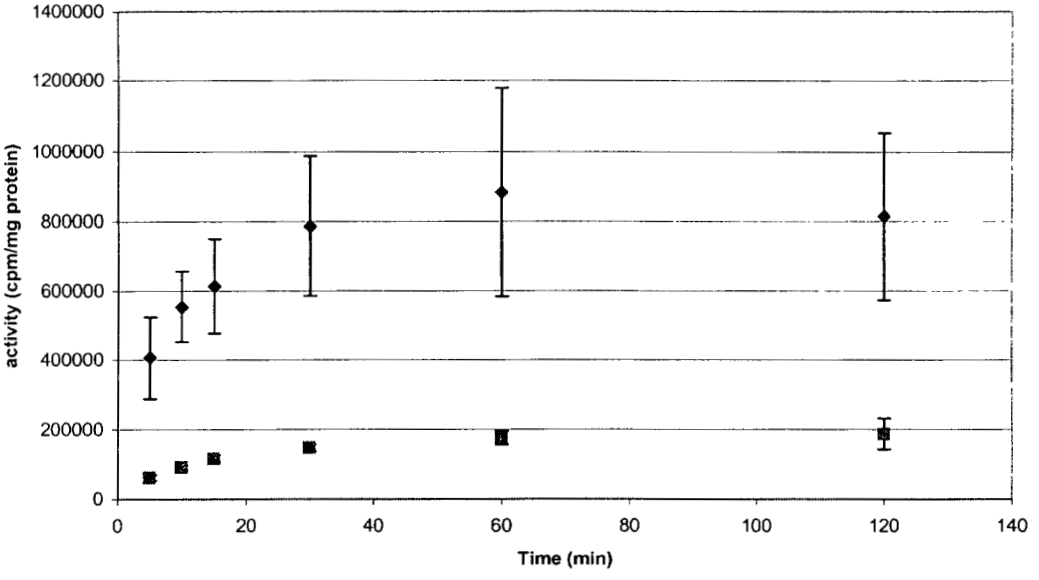

Figure 2. Uptake of ^{99m}Tc-labelled rhLf into RT112 bladder cancer cells. (◆) total cell associated uptake; (■) internalised uptake, in cpm/mg protein. The error bars show the standard deviation of at least three points.

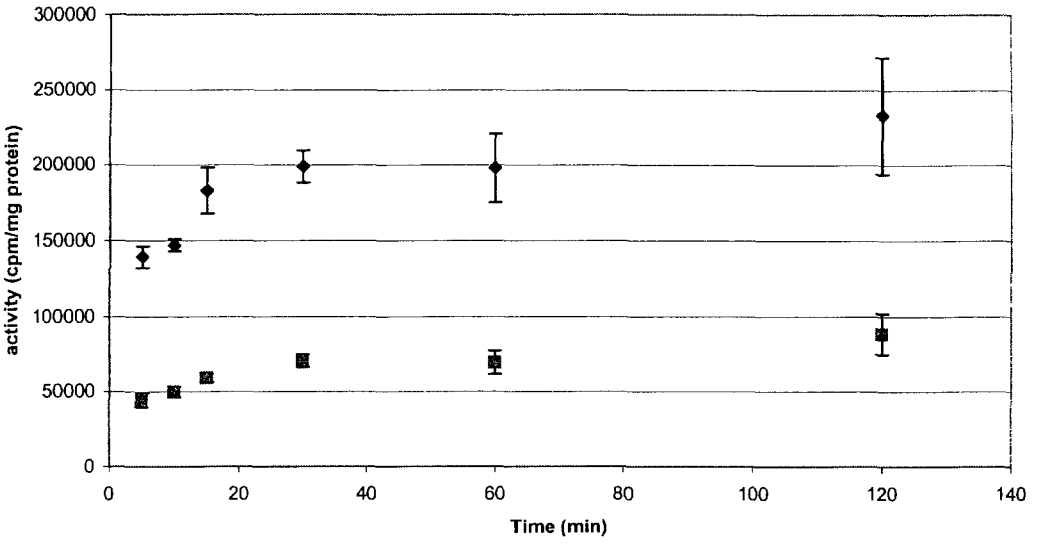

Figure 3. Uptake of ^{99m}Tc-labelled rhLf into COLO205 colo-rectal cancer cells. (◆) total cell associated uptake; (■) internalised uptake, in cpm/mg protein. The error bars show the standard deviation of at least three points.

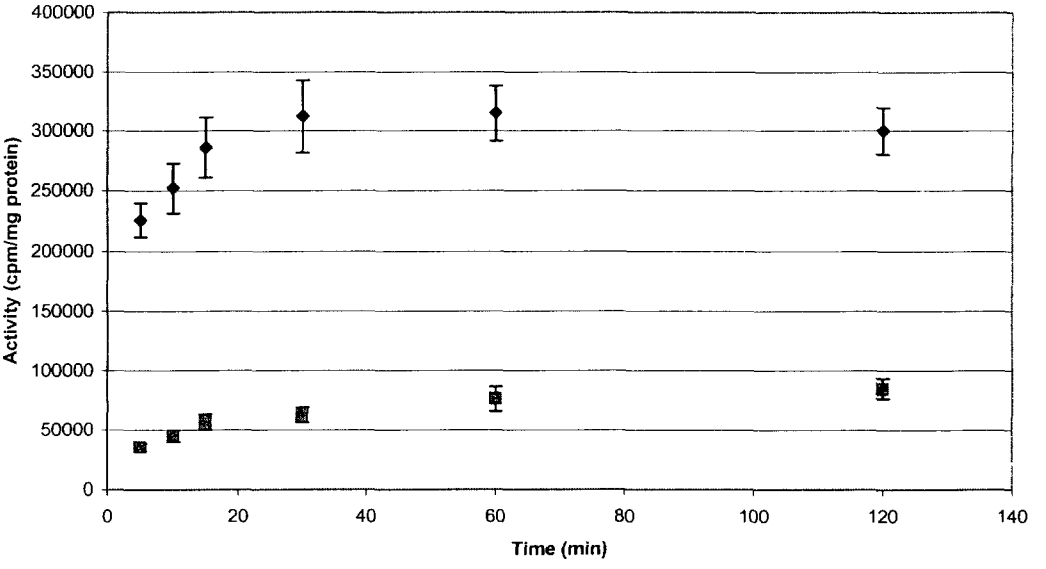

Figure 4. Uptake of ^{99m}Tc-labelled rhLf into PC3 prostate cancer cells. (◆) total cell associated uptake; (■) internalised uptake, in cpm/mg protein. The error bars show the standard deviation of at least three points.

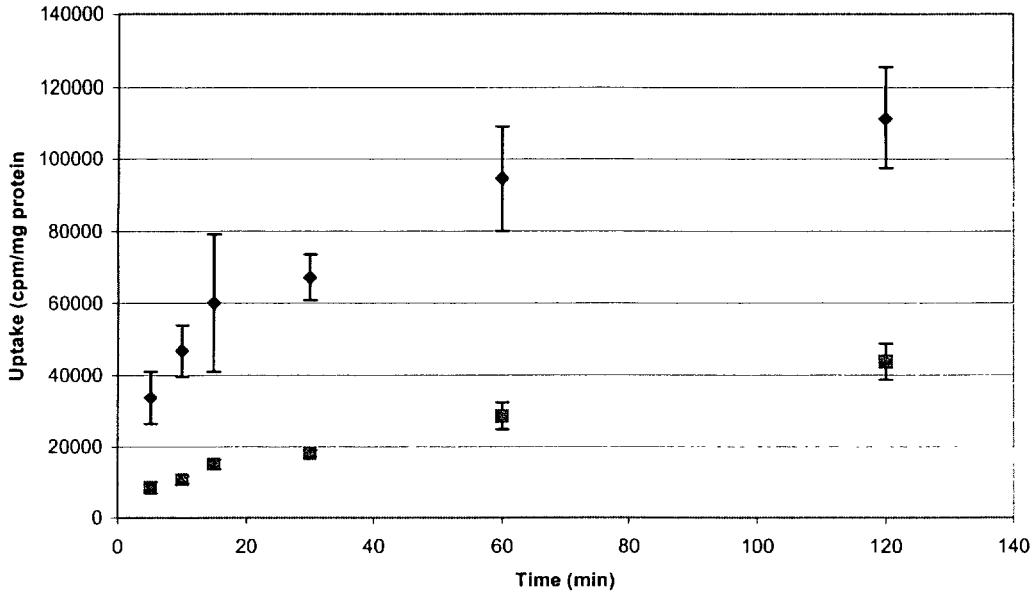

Figure 5. Uptake of ^{99m}Tc-labelled rhLf into PNT2-C2 human prostate cells. (◆) total cell associated uptake; (■) internalised uptake, in cpm/mg protein. The error bars show the standard deviation of at least three points.

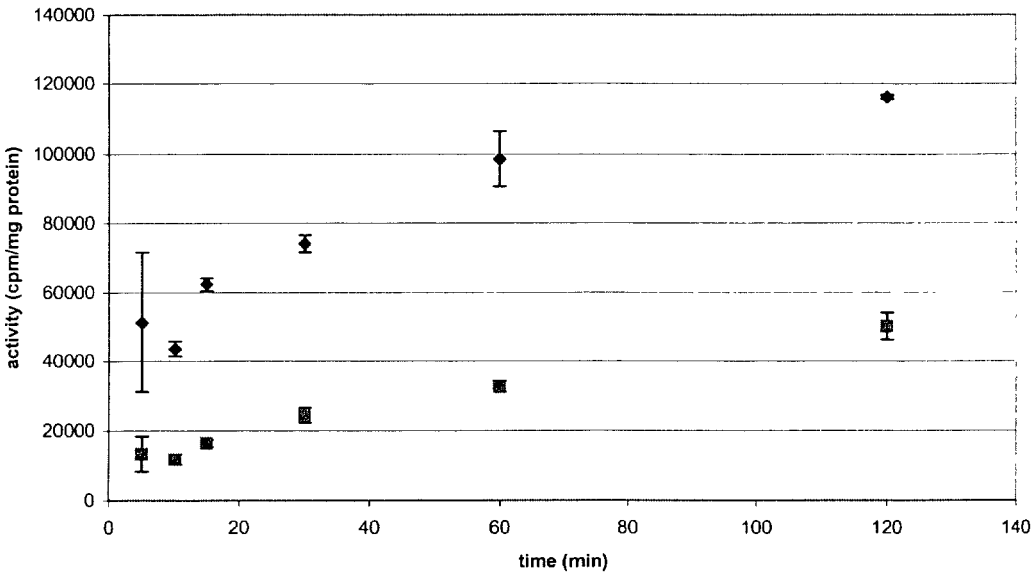

Figure 6. Uptake of ^{99m}Tc-labelled rhLf into MCF7 human breast cancer cells. (◆) total cell associated uptake; (■) internalised uptake, in cpm/mg protein. The error bars show the standard deviation of at least three points.

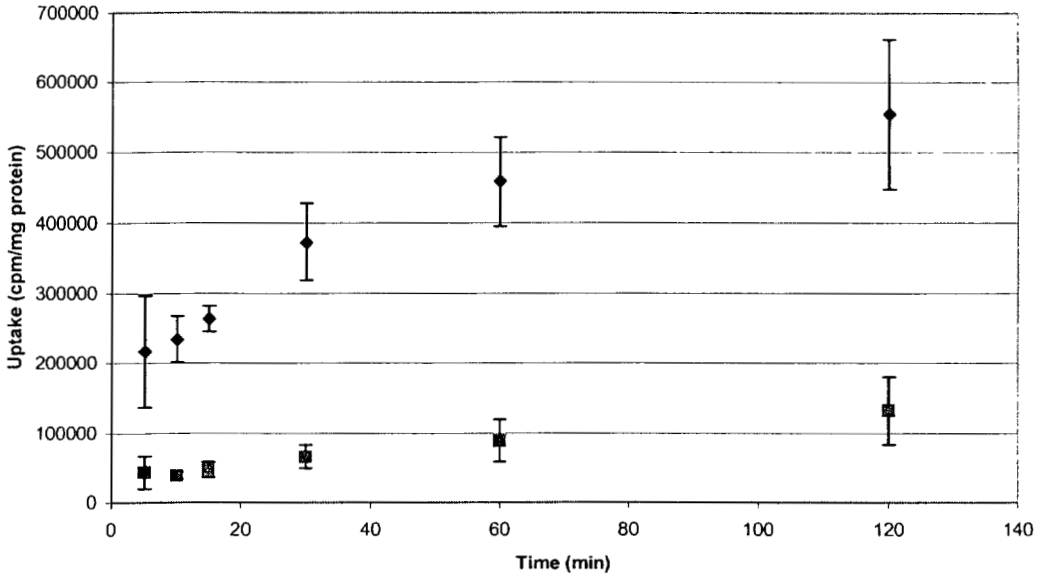

Figure 7. Uptake of ^{99m}Tc-labelled rhLf into SK-N-SH human brain cancer cells. (◆) total cell associated uptake; (■) internalised uptake, in cpm/mg protein. The error bars show the standard deviation of at least three points.

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Antibiotic Tolerance Induced by Lactoferrin in Clinical *Pseudomonas aeruginosa* Isolates from Cystic Fibrosis Patients

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Lactoferrin-induced cell depolarization and a delayed tobramycin-killing effect on *Pseudomonas aeruginosa* cells were correlated. This antibiotic tolerance effect (ATE) reflects the ability of a defense protein to modify the activity of an antibiotic as a result of its modulatory effect on bacterial physiology. *P. aeruginosa* isolates from cystic fibrosis patients showed higher ATE values (≤6-fold) than other clinical strains.

Aminoglycoside antibiotics have been shown to be effective in treating *Pseudomonas aeruginosa* lung infections of cystic fibrosis (CF) patients (8). However, medical experience shows that in vitro aminoglycoside-susceptible *P. aeruginosa* may persist in an infected lung despite appropriate antibiotic treatment, indicating a dissociation between in vitro and in vivo antibiotic susceptibility (13). This paradoxical phenomenon reflects the influence of environmental, bacterial, and host factors that are not present in in vitro susceptibility tests. For example, antibiotic susceptibility is modified in vitro and in vivo by bacterial factors, including biofilm formation (10), or by some physiological changes, such as membrane depolarization of anaerobically grown bacteria that results in aminoglycoside resistance (4). Some host defense compounds (e.g., lactoferrin) have also been reported as modifying the in vitro antibiotic susceptibility of bacteria and yeasts (1, 3, 9). Despite the fact that some of these defense compounds increase during the infection, there are few studies exploring their influence on antibiotic activity.

Human lactoferrin (hLf) is an innate defense protein, mainly present in mucosal secretions, that is found at high concentrations (~0.9 mg/ml) in the sputa of CF patients infected by *P. aeruginosa* (6). We have recently reported that this protein induces a decrease in the transmembrane electrical potential of bacteria and yeasts (14, 15). Since aminoglycoside uptake is dependent on electrical potential of the bacterial membrane, we attempted to determine whether membrane depolarization induced by lactoferrin may decrease susceptibility to tobramycin. If this scenario were so, that effect could partly explain the apparently different in vivo and in vitro susceptibilities of *P. aeruginosa* strains isolated from CF patients.

The strains studied included *P. aeruginosa* ATCC 9027, *P. aeruginosa* FRD1 (cystic fibrosis isolate *mucA22* [Alg^+]) and *P. aeruginosa* FRD1131 (*mucA22 algD*::Tn*501-33* [Alg^-]) (a gift from D. E. Ohman, Virginia Commonwealth University). Fourteen clinical isolates of *P. aeruginosa* from the sputum of different CF patients were provided by F. Baquero (Hospital Ramón y Cajal, Madrid, Spain), C. Bousoño (Central Hospital of Asturias, Oviedo, Spain), and I. Planells (Vall d'Hebron Hospitals, Barcelona, Spain); six isolates from non-CF patients were also included in the study. The clinical isolates were identified by the API 20NE system. Recombinant human lactoferrin (rhLf) was provided by Ventria Bioscience (Sacramento, Calif.). Gentamicin and tobramycin were purchased from Sigma (St. Louis, Mo.). MICs were determined by the NCCLS microdilution method (12) with Mueller-Hinton medium (Difco) and inocula of 5×10^5 CFU/ml. The MIC was defined as the lowest concentration at which there was no visible growth after 24 h of incubation at 37°C.

Time-kill assays were carried out with all *P. aeruginosa* strains as described previously (14). Briefly, the bacterial suspensions (10^6 CFU/ml) in 10 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl were preincubated with rhLf (0.9 mg/ml) for 10 min before the addition of the antibiotic (at the MIC). Duplicate samples were then removed every 30 min during 3.5 h, and dilutions were plated onto Mueller-Hinton agar to obtain a viable count. Bacterial viability ($\log_{10}$ CFU/ml) was plotted against time for each experiment. The antibiotic tolerance effect (ATE) was defined as the difference in time (in hours) between the rhLf-treated and untreated suspensions for the bacterial counts to decrease 1 log unit below that measured immediately after the addition of antibiotic. This effect was calculated by the following equation: $ATE = T - C$, where T is the time required for the host factor (rhLf)-exposed cell suspension to decrease 1 $\log_{10}$ below the count observed immediately after the addition of the drug (tobramycin or gentamicin) and C is the time required for the untreated suspension to decrease 1 $\log_{10}$ below the count observed immediately after the addition of the drug.

The membrane potential was determined by using the fluorescent probe bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4$; Molecular Probes, Eugene, Oreg.) (3) as described previously (14). Briefly, exponential-phase bacteria were washed and resuspended (10^5 CFU/ml) in 10 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl and then incu-

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TABLE 1. Antibiotic tolerance induced by lactoferrin in *P. aeruginosa* strains

P. aeruginosa strain[a]	Parameter for tobramycin (parameter for gentamicin)[b]			
	MIC (μg/ml)	*T* (h)	*C* (h)	ATE (h) (mean ± SD)
ATCC 9027	1	1.4	0.9	0.5 ± 0.1
CI strains				
O1082	1 (2)	0.9 (1.7)	0.3 (1)	0.4 ± 0.1 (0.7 ± 0.1)
O1090	1	1.6	0.8	0.6 ± 0.1
O1100	0.25	0.7	0.4	0.3 ± 0.2
O1160	1	1.2	0.6	0.6 ± 0.1
O2785	0.5	1.4	0.7	0.4 ± 0.1
O4001	1 (2)	1.6 (2.3)	1.1 (1.5)	0.5 ± 0.1 (0.8 ± 0.1)
CF strains				
B129	1	3	1.5	1.5 ± 0.2
B191	1	1.9	0.4	1.5 ± 0.3
B218	1	2.4	0.8	1.6 ± 0.2
B231	1	2.4	0.9	1.5 ± 0.1
B529	2	2.8	1.4	1.4 ± 0.1
B566	0.5	1.7	0.5	1.2 ± 0.2
O50850	2 (2)	2.1 (2.4)	0.8 (0.9)	1.3 ± 0.3 (1.5 ± 0.2)
O52733	2 (2)	2.4 (2.7)	1.1 (1.4)	1.3 ± 0.3 (1.3 ± 0.3)
O65711	1	2.2	0.6	1.6 ± 0.1
O90401	1	2.6	0.7	1.9 ± 0.5
O93814	4	2.9	1.2	1.7 ± 0.5
M19915	1	1.8	0.7	1.1 ± 0.1
M65323	1	2.3	1	1.3 ± 0.2
M99130	1	1.7	0.3	1.4 ± 0.1

[a] CI strains, clinical isolates from different sources. CF strains, clinical isolates from sputum of CF patients.

[b] *T*, time required for the host factor-exposed cell suspension to decrease 1 $\log_{10}$ below the count observed immediately after the addition of the drug; *C*, time required for the untreated suspension to decrease 1 $\log_{10}$ below the count observed immediately after the addition of the drug. Also determined were the ATEs of the MICs of tobramycin and gentamicin in the presence of lactoferrin (0.9 mg/ml).

bated with rhLf (0.9 mg/ml) at 37°C for 15, 30, 60, 90, 120, and 180 min. Samples were then reincubated for an additional 10 min with $DiBAC_4$ (0.4 μM, final concentration) (3) and analyzed by cytofluorometry.

All experiments were performed at least in triplicate. Results were analyzed by the Mann-Whitney U test. A *P* value of <0.05 was considered significant.

The *P. aeruginosa* strains studied were sensitive to the aminoglycosides tobramycin and gentamicin in a conventional MIC test (Table 1). Since the in vitro bactericidal activity of lactoferrin was inversely related to the extracellular concentrations of NaCl and divalent cations (14, 15), the evaluation of the MIC of rhLf and combination effects (i.e., fractional inhibitory concentration) of rhLf and the antibiotic was not possible by using standard microbiological media. Consequently, time-kill assays were performed in Tris buffer containing NaCl. Figure 1 shows illustrative results from time-kill assays corresponding to *P. aeruginosa* clinical isolate O4001 and CF strain B129 isolated from a diabetic foot ulcer and the sputum of a CF patient, respectively. The killing by tobramycin in rhLf-pretreated cells was not immediate. Table 1 summarizes the ATE values of 21 *P. aeruginosa* strains, including 14 clinical isolates from different CF patients attended in three hospitals located in different geographical areas. The comparison of the results obtained by using individual MICs of tobramycin or gentamicin and rhLf (0.1 to 1 mg/ml) showed that the ATE values of CF isolates were significantly ($P < 0.05$) higher (≤6-fold) than those calculated for the non-CF isolates. This difference was apparently not related to the controversial affinity of aminoglycosides to the exopolysaccharide alginate, because both *P. aeruginosa* FRD1 (Alg^+) and its nonmucoid derivate *P. aeruginosa* FRD1131 (Alg^-) showed similar ATE values (data not shown). The influence of alginate on this effect, if any, could be more relevant in the cystic fibrotic lung, where alginate-overproducing *P. aeruginosa* communities are organized in biofilms exhibiting increased tobramycin resistance (7, 10). In this case, the exposure of this pathogen to suboptimal antibiotic concentrations as a consequence of the aminoglycoside binding to the alginate matrix could increase the ATE values induced by hLf with respect to those observed in our assays with planktonic cells.

After rapid electrostatic binding, the aminoglycoside uptake in *P. aeruginosa* has been reported to occur in two phases: a slow-uptake phase that depends on the magnitude of the bacterial transmembrane electrical potential and a subsequent rapid-uptake phase due mainly to the membrane permeabilization caused by the antibiotic (2, 4). Although the accumulation of tobramycin in *P. aeruginosa* cells was not verified in the present study, it is well known that the depolarization of the membrane potential implies less uptake of the aminoglycosides and consequently a decreased bacterial susceptibility (4). Representative data from experiments performed with *P. aeruginosa* clinical isolate O4001 and CF strain B129 exposed to rhLf at previously indicated times are shown in Fig. 1. A high percentage (>80%) of fluorescent cells, indicative of rapid membrane depolarization, was observed. Similar data were obtained with all *P. aeruginosa* isolates exposed to rhLf, as well as in control assays when the cells were treated with carbonyl

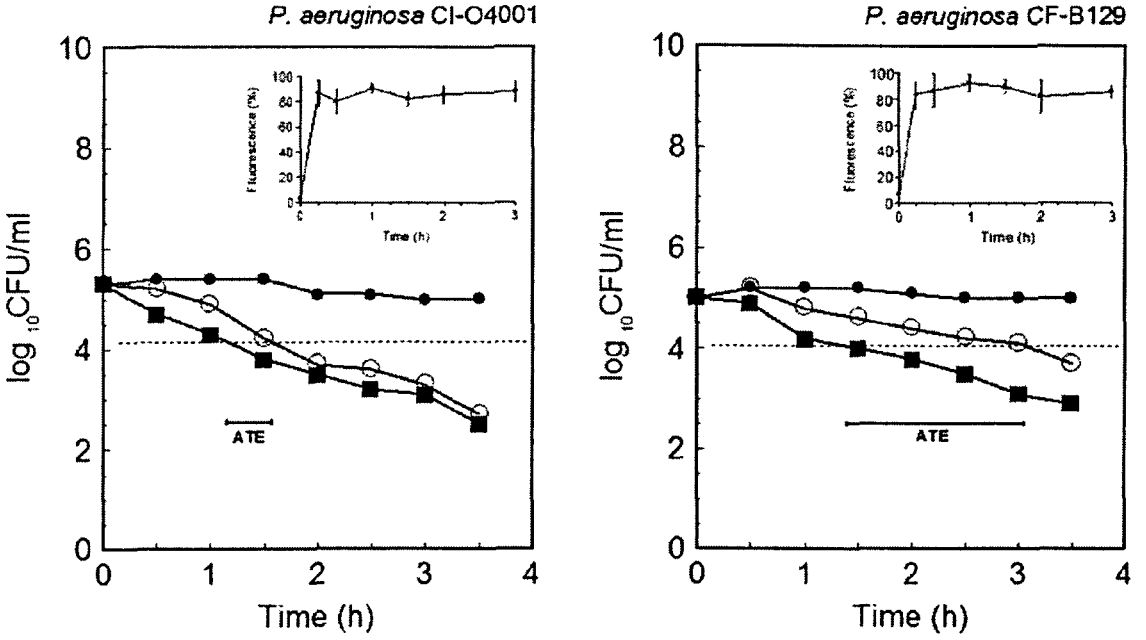

FIG. 1. Time-kill assay curves for tobramycin in the presence of lactoferrin. *P. aeruginosa* cells were incubated with 0.9 mg of lactoferrin (●)/ml, 1 μg of tobramycin (■)/ml, or 0.9 mg of rhLf/ml and 1 μg of tobramycin/ml (○). Results are the means from duplicates of at least three independent assays. The antibiotic tolerance effects are indicated (bars). The effect of lactoferrin on the membrane potential of *P. aeruginosa* was determined by flow cytometry using the fluorescent probe $DiBAC_4$ (3) (insets). Percentages of fluorescence (fluorescent cells) correspond to bacteria with membrane depolarization. CI, clinical isolate. CF, clinical isolate from sputum of a CF patient.

cyanide *m*-chlorophenylhydrazone (10 μM) (data not shown). Since we have recently reported that hLf decreases the bacterial membrane potential without a permeabilizing effect (14), we speculate that the depolarization inhibited the antibiotic uptake during the slow-uptake phase, as the inability of tobramycin to decrease the number of *P. aeruginosa* cells seems to reflect. This delayed phase was followed by a rapid loss of cell viability that apparently corresponded to the later rapid-uptake phase (2), since it was partially inhibited by the presence of 1 mM $MgCl_2$ (data not shown) as described previously (5).

Aminoglycoside activity against *P. aeruginosa* in the sputum of CF patients is significantly decreased (10- to 25-fold) by host and bacterial factors that are still not fully understood (11). The identification of these factors may therefore contribute to improving antibiotic dosing regimens to eradicate *P. aeruginosa* from these patients. Based on our results, we hypothesize that lactoferrin could be a host factor that decreases the efficacy of tobramycin in vivo. Since the ATE value was tobramycin concentration dependent (data not shown), it is possible that this effect might be partially overcome when high drug concentrations are achieved at the site of the infection (e.g., in inhalatory therapy).

In conclusion, this study shows that lactoferrin induces a transitory tolerance to tobramycin on *P. aeruginosa* significantly in CF clinical isolates. Although it is known that environmental parameters and bacterial and host factors may modulate antibiotic activity, our results show for the first time that a host defense protein is able to modify antibiotic susceptibility as a consequence of its modulatory effect on bacterial physiology.

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Ventria Testimony Before The Subcommittee on Rural Enterprises, Agriculture, and Technology, United States House of Representatives, June 29, 2005

Hearing Name:	Different Applications for Genetically Modified Crops
Committee:	Subcommittee on Rural Enterprises, Agriculture, and Technology
Date:	Wednesday, June 29, 2005

Prepared Remarks of Mr. Scott Deeter
President and CEO Ventria

Good afternoon Chairman Graves (R-MO), Members of the Committee, Ladies and Gentlemen. My name is Scott Deeter and I am President & CEO of Ventria Bioscience. I appreciate the opportunity to address the Committee on behalf of Ventria Bioscience. I will briefly describe the company, our technology and our products and would be happy to answer any questions.

First, let me provide an introduction to Ventria Bioscience. Ventria Bioscience is a plant-made pharmaceutical company that utilizes rice and barley as a factory to produce biologic products. Ventria's initial products provide human health benefits, however the Company's technology has the potential to address many challenges faced by other sectors of the economy including animal health, energy and industrial processing.

Ventria was founded with the support and guidance of several leaders in biotechnology and agribusiness who form the Company's Board of Directors. Ventria's Chairman is Thomas N. Urban, Jr. former Chairman and CEO of Pioneer Hi-Bred International. Other Board members include William J. Rutter, Ph.D. and Pablo Valenzuela, Ph.D., who were Co-Founders of Chiron; William H. Rutter, an attorney by training and an entrepreneur; William W. Crouse, a limited partner of Healthcare Ventures; Dean Hubbard, Ph.D. President of Northwest Missouri State University and Melvin D. Booth, former President of MedImmune, Inc. and Human Genome Sciences, Inc. These industry leaders have committed their resources, their time and their talents to realize the vision of improving healthcare on a global basis utilizing the tools of modern biotechnology combined with the industrial might of American agriculture.

The company's core technology is a highly efficient and unsurpassed method of producing biological products in the seed of self-pollinating rice and barley. This technology was discovered in collaboration with University of California as well as other leading research institutions in the United States.

Ventria believes this technology will lead to more affordable medicines for a much broader patient population than what is possible with conventional biopharmaceutical production technology today. Ventria's technological innovation results in a substantial improvement in the economics of biopharmaceutical production. For instance, the capital investment required for Ventria to produce 500 kilograms is estimated to be $4 million. As a comparison, to produce the same amount using conventional technology, such as mammalian cell culture, would require capital investment exceeding $125 million, a more than 30 fold increase. In addition, the operating costs of Ventria's technology are less than 10% of the conventional technology.

There are several reasons for this economic advantage. First, Ventria has been able to achieve extraordinarily high yields of the product in the seed of rice and barley. Second, barley and rice are self-pollinating crops that can easily achieve the necessary geographic isolation from their food crop counterparts to eliminate concerns of cross contamination with the food supply. Third, because these crops can be stored in ambient conditions for up to two years without degradation, they allow for continuous operation of a processing facility, thereby increasing capacity utilization and reducing cost. Fourth, because rice and barley are safe for human consumption, they are ideal for products that can be delivered orally, thereby eliminating the need for expensive separation technology that is required by conventional systems to remove infectious or toxic contaminants. These advantages pave the way for a paradigm shift in biopharmaceutical production for the benefit of patients worldwide.

As an illustration of the strength of Ventria's technology, I would like to describe some of the human health products in development. Ventria's first two human health products are proteins called Lactiva™ and Lysomin™. These two proteins are found naturally in mother's milk, saliva and tears and they have been suggested to contribute to the improved health status that has been widely reported for breast fed children when compared to their bottle fed counterparts. These proteins are part of the reason why breast feeding is the best form of nutrition for infants and is highly recommended by most pediatricians.

Ventria currently produces Lactiva™ and Lysomin™ in the seed of rice through contract relationships with selected and well trained growers. Ventria's field production is regulated under a permit that is issued by the United States Department of Agriculture's Animal and Plant Health Inspection Service ("APHIS"). In fact, last year alone, Ventria's field location was inspected eight times by APHIS inspectors. Once harvested the seed is pulverized into a powder and transported to the processing facility where the final product is isolated into either a concentrate or isolate

The United States Food and Drug Administration ("FDA") has regulatory authority over Ventria's products for human health. As part of Ventria's pre-market activities, we reviewed the safety of Lactiva™ and Lysomin™ with a panel of scientific and medical experts that have unanimously concluded that these products are Generally Recognized as Safe ("GRAS") for human consumption. The results of the panel review were

summarized and submitted to FDA where they are awaiting clearance prior to commercial sales for human health.

There are several products being developed by Ventria that will incorporate Lactiva™ and Lysomin™. One product has been developed for children suffering from acute diarrhea. The World Health Organization estimates that 1.9 million children under the age of 5 die annually due to diarrhea. To address this crisis, Ventria added Lactiva™ and Lysomin™ to an oral rehydration solution, which is a common first line therapy given to children suffering from diarrhea. By adding Lactiva™ and Lysomin™, Ventria believes it can improve the recovery rate and reduce the severity or duration of diarrhea in these children. This hypothesis is the basis of a recently completed study in Peru with 150 children suffering from acute diarrhea. Ventria expects the results of this study to be published shortly. Ventria's production technology enables the cost effective addition of Lactiva™ and Lysomin™ to oral rehydration solution for the benefit of millions of children globally.

Ventria is also exploring the use of Lactiva™ and Lysomin™ for the prevention of diarrhea in the military. During Operation Iraqi Freedom, 70% of deployed troops suffered a diarrheal attack and 43% reported decreased job performance as a result of this attack. During the Viet Nam War, it has been reported that hospitalizations due to diarrhea were four times more prevalent than malaria. This is a silent enemy attacking American troops. Ventria has set its goal to reduce the diarrheal attack rate by 50% with the preventive administration of Lactiva™ and Lysomin™. If we achieve our objective, it would improve military morale, efficiency, and manpower. In terms of manpower productivity alone, this may pay for itself due to the cost effectiveness of Ventria's technology. Incidentally, this is a similar problem to that experienced by the millions of Americans who travel overseas.

Another use of Lactiva™ that is being developed is for the management of inflammatory bowel disease, or IBD. IBD afflicts over one million Americans and over four million people worldwide. IBD is an extremely debilitating disease that causes severe abdominal pain, weight loss, poor absorption of nutrients and chronic gastrointestinal ulcers. Ventria is testing the potential for Lactiva™ to improve the quality of life for the millions with this disease.

Ventria is also working with University of Cincinnati to develop a treatment for chronic lung infections caused by Pseudomonas, which is the leading cause of death for patients suffering from Cystic Fibrosis. Ventria and our collaborators have shown successful inhibition of this infection and we are jointly planning a pre-clinical program to further develop this product.

Recently, Ventria was the recipient of an SBIR grant from National Institutes of Health, National Institute on Aging relating to the use of one of Ventria's products to inhibit biofilms constructed by pathogenic bacteria. These types of infections affect more than 10 million Americans annually. Infections that are protected by biofilms are 100 to 1,000

times more resistant to antibiotics, so it is important to inhibit the formation of these biofilms before they can establish themselves at the wound site. Ventria has worked with scientists from University of Iowa and Howard Hughes Medical Institute to develop a natural human protein that has been shown to inhibit the ability of pathogens to construct these biofilms. Using its plant -made pharmaceutical technology Ventria produced and purified this protein and has shown the effective inhibition of biofilm formation. With the SBIR grant, Ventria will further develop this product with the goal of improving patient recovery by reducing the establishment of biofilms that lead to antibiotic resistant pathogens.

This concludes my testimony on behalf of Ventria Bioscience. I would like to thank Chairman Graves and the Committee members for your kind attention and would be happy to answer any questions you may have.

5

Ventria Claims the Pharmaceutical Use of Recombinant Human Lactoferrin in Treating Human Disease

Ventria's own website currently indicates that the Company's intended use of Recombinant Human Lactoferrin is to treat diseases including acute diarrhea, fungal infections and topical infections, as well as inflammation. The website also claims a wide range of biologic activity for lactoferrin consistent with Ventria's proposed ***pharmaceutical*** applications.

In a glossary published on Ventria's website, the company defines its own terms for its products - ***Biopharmaceuticals***:

Output Traits: In agricultural biotechnology, input traits are traits that improve the agronomic performance of the plant (I.e. RoundUp Ready(R) Corn). Output traits are traits that change the way the plant is used. In this case, ***Ventria's output traits are biopharmaceuticals***.

Pharmaceutical: Of or pertaining to the knowledge or art of pharmacy, or to the art of preparing ***medicines*** according to the rules or formulas of pharmacy; as, ***pharmaceutical preparations***. ["Biopharmaceuticals" are pharmaceuticals produced in biologic systems]

Plant-Made Pharmaceuticals: The art of preparing ***medicines*** according to the rules or formulas of pharmacy through the use of plants.

Therapeutic Proteins: A protein, of or pertaining to the healing art; concerned in discovering and applying ***remedies for diseases; curative***.

In a presentation to the USDA's Advisory Committee on Biotechnology & 21st Century Agriculture, Scott Deeter, Ventria's CEO, described his Company's vision of using plants as the ***"host for the manufacture of the active ingredient for a drug"***. He also clearly indicated Ventria's focus on treating acute respiratory infections and diarrheal diseases. **(Attached)**

Ventria is represented as a company that uses rice as a "factory for producing human ***medicine***." Scott Deeter, Ventria's CEO, asserts that lactoferrin and lysozyme are intended to treat diseases such as anemia and diarrhea and states, "Ventria sees itself -- as a biotechnology company hard at work on ***medical*** products that could save lives."

"Bioengineered rice takes center of debate over using food crops to grow drugs." *San Jose Mercury News, April 16, 2004* **(Attached)**

Ventria CEO Scott Deeter clearly asserts that Ventria's objective for lactoferrin is an "orally administered ***biopharmaceutical***" (drug) which will be used to treat human disease including bacterial and viral infections, anemia, and diarrhea.

Interview with *Rehydrate.org, May 27,2004*

Ventria CEO Scott Deeter asserts that his lactoferrin and lysozyme "would be the first genetically engineered plant-produced ***pharmaceuticals*** to reach the market." He further states that Ventria's rice is intended to ***"treat severe diarrhea"*** and is ***"not intended as food"***.

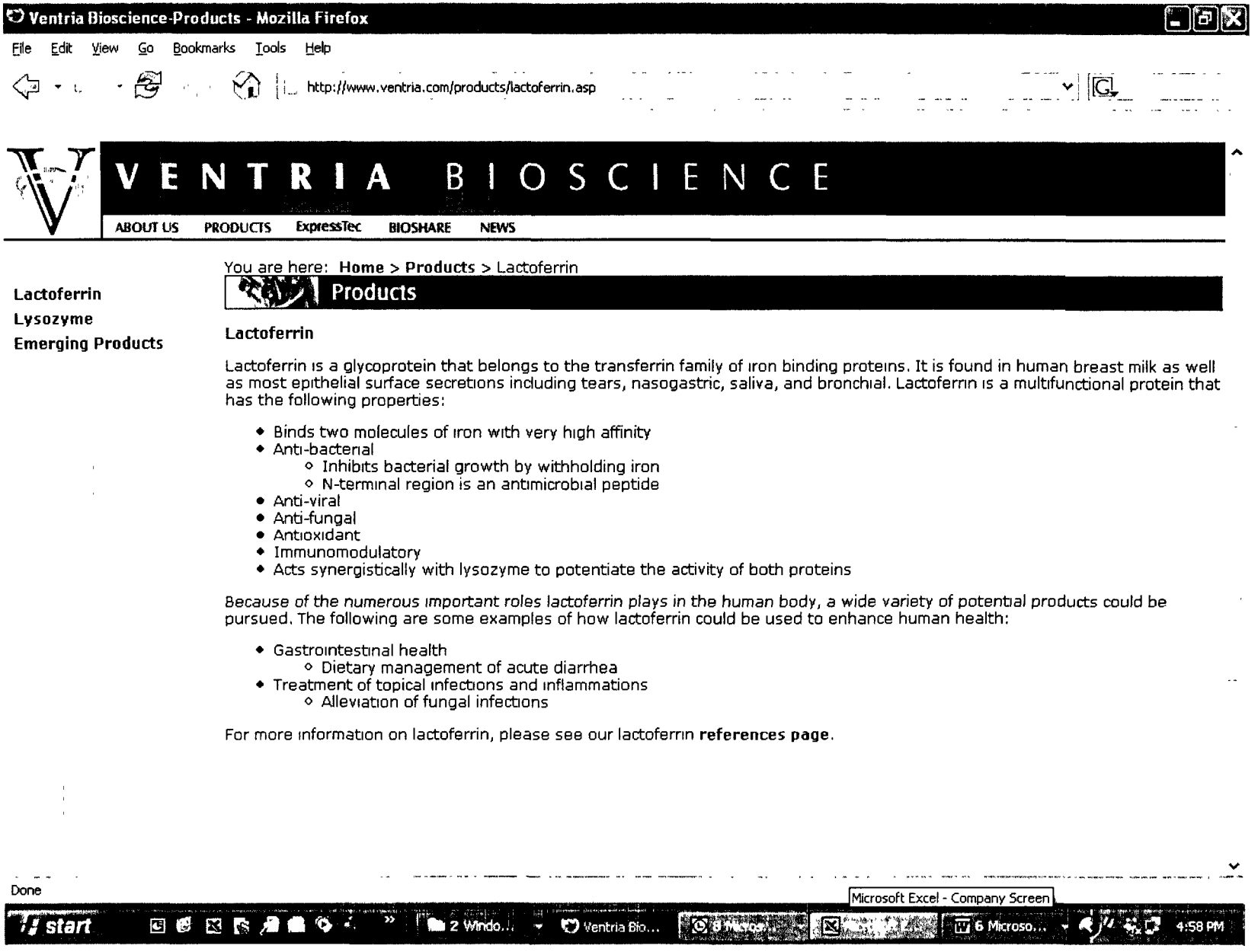
"Biotech company cultivates new field." *Sacremento Bee, January 25, 2004*

In a scientific periodical, Ventria's lactoferrin and lysozyme are described as "**drugs**" with Ventria's CEO Scott Deeter making an unsubstantiated claim that "***Ventria's products have the potential to save the lives of 2 million children a year.***"

"California OKs GM Pharm Crops." *The Scientist, April 8, 2004*

In previous interviews, Ventria has clearly stated its objectives and intentions to develop its products as pharmaceuticals for the treatment of disease. In an interview with the London newspaper *The Independent*, Ventria directly stated this intent. According to *The Independent,* Ventria says that its plants "will become 'factories' that manufacture ***therapeutic proteins*** to combat ***life-threatening illnesses***". It adds that "plants improved through the use of biotechnology" can produce them "***for innovative treatments for diseases such as cancer, HIV, heart disease, diabetes, Alzheimer's disease, kidney disease, Crohn's disease, cystic fibrosis and many others.***"

"GM Rice To Be Grown For Medicine." *The Independent, February 1, 2004*



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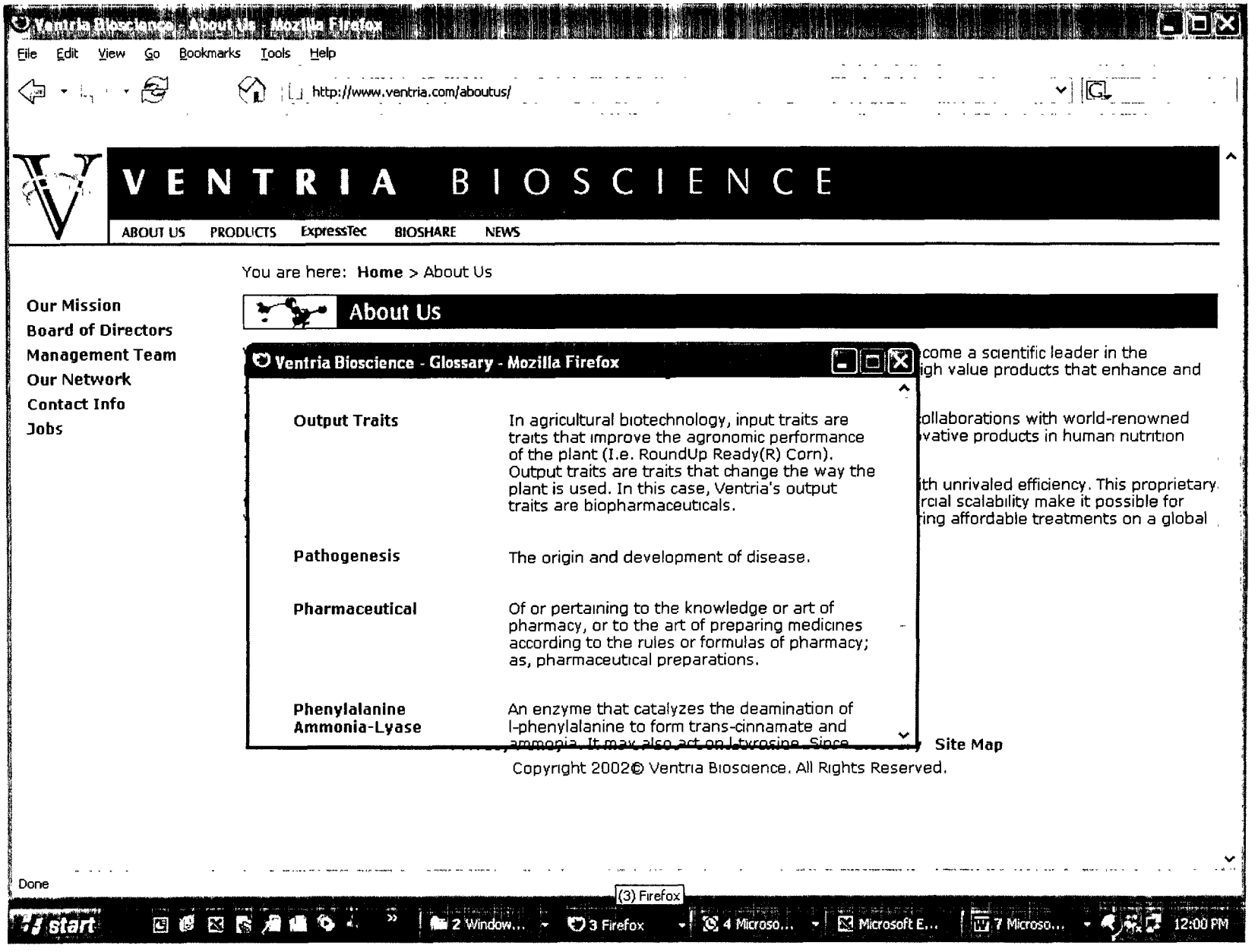

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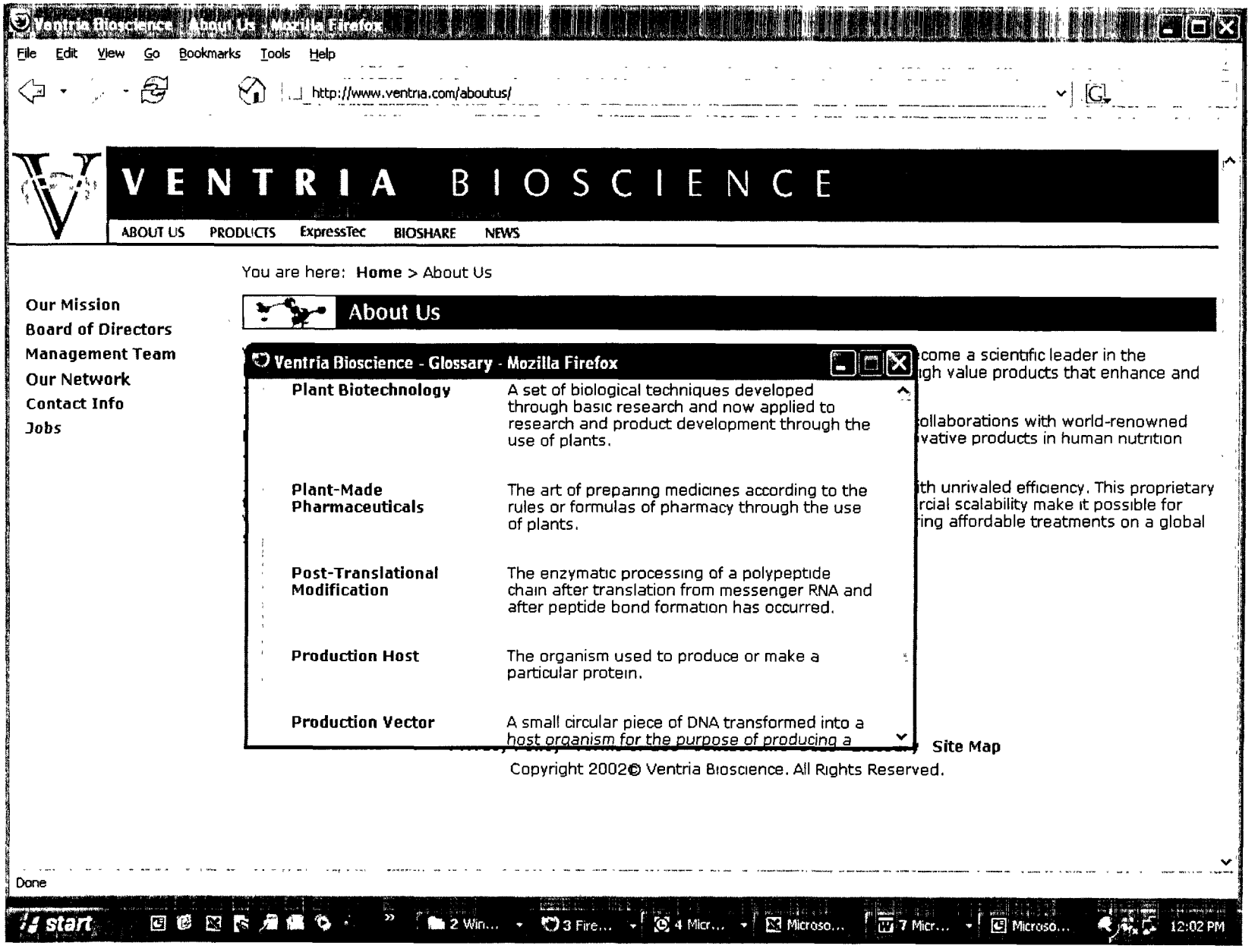

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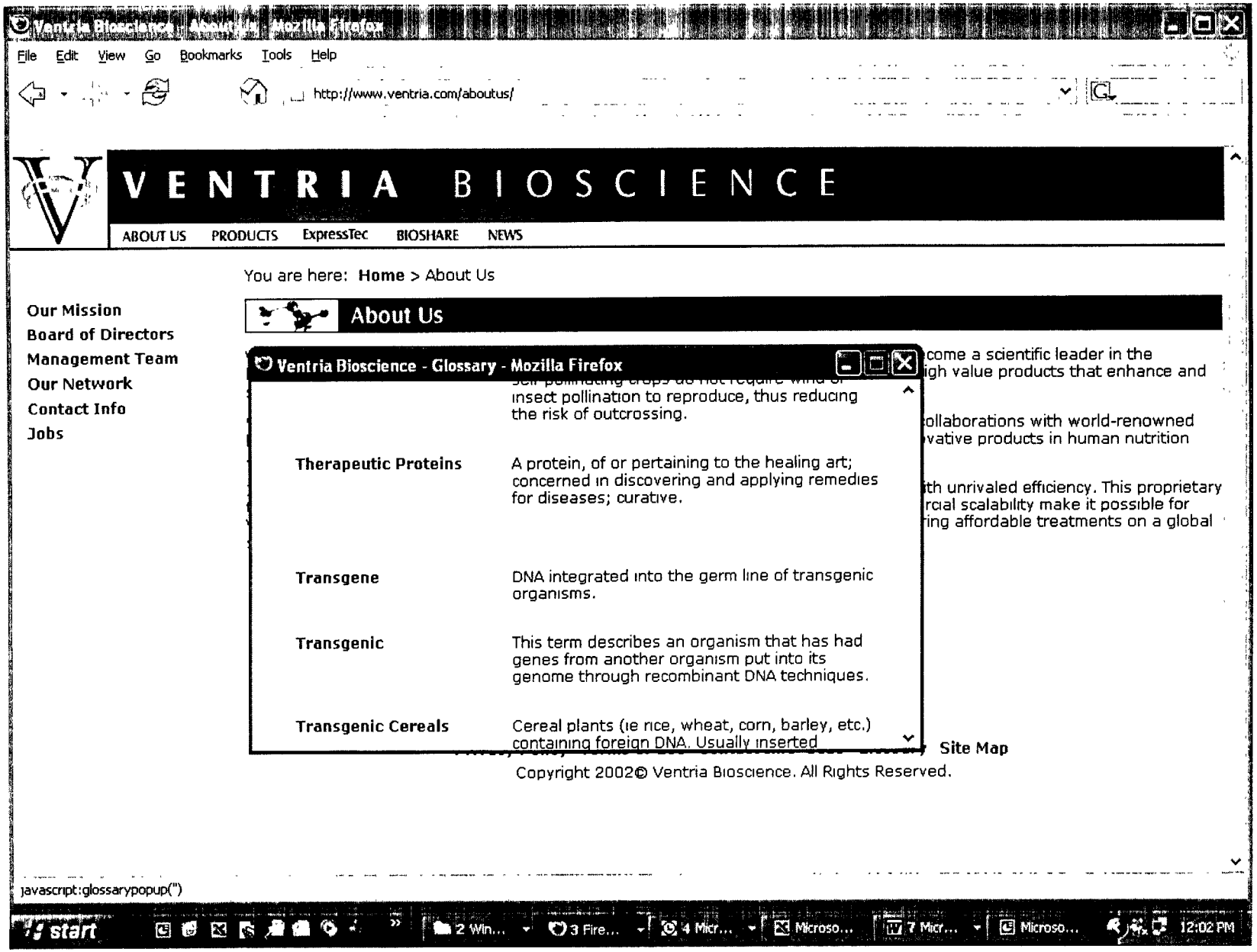


July 18, 2005

Ventria Presentation to the USDA's Advisory Committee on Biotechnology & 21st Century Agriculture

VENTRIA BIOSCIENCE

Plant-made Pharmaceuticals & Industrials:

Expectations & Realities

Scott Deeter

Ventria Bioscience

June 17, 2003

http://www.usda.gov/agencies/biotech/ac21/meetings/mtg_june03/ac21_mtg_june03.html

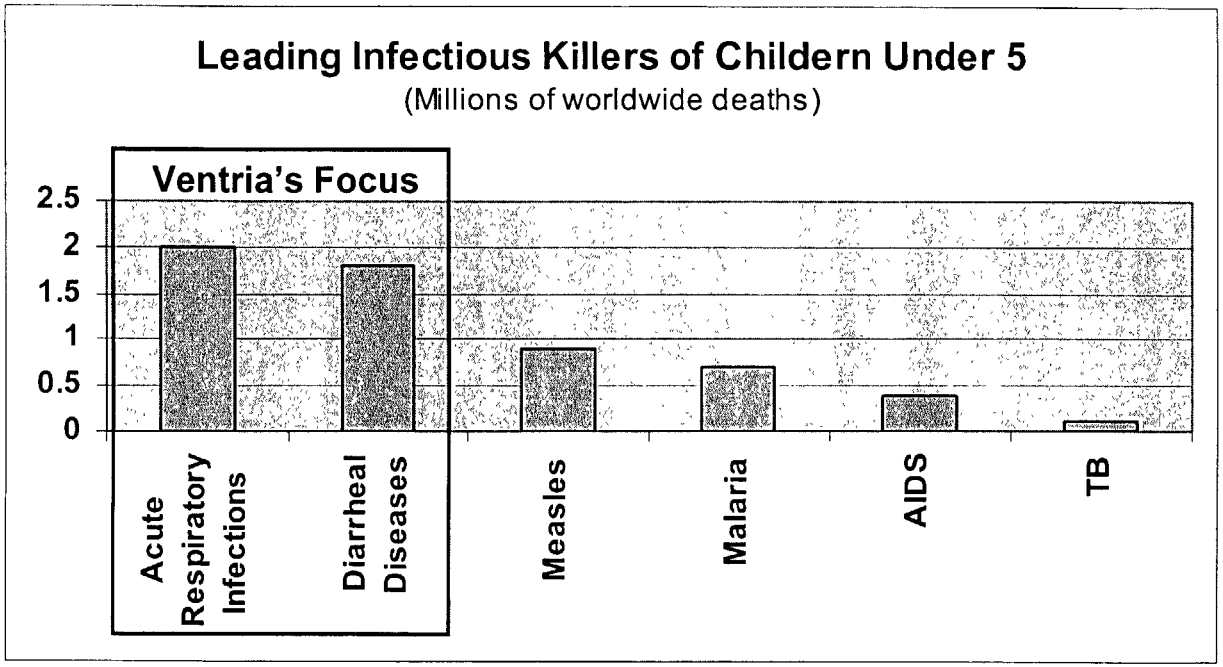
What are PMPs and PMIPs?

- Plant-made Pharmaceuticals (PMPs)
  - New category of products
  - Plant is the host for the manufacture of the active ingredient for a drug.
  - Following harvest, plant material is processed to recover and purify the active ingredient.
  - Active ingredient manufacture and marketing is regulated by FDA

- Plant-made Industrial Products (PMIPs)
  - New category of products
  - Plant is the host for the manufacture of an industrial product, such as an industrial enzyme.
  - Following harvest, plant material is processed to recover and purify the industrial compound.

http://www.usda.gov/agencies/biotech/ac21/meetings/mtg_june03/ac21_mtg_june03.html

Global Health Problems



Source: *World Health Organization*

Bioengineered rice takes center of debate over using food crops to grow drugs

April 16
San Jose Mercury News

There are two very different views of Ventria Bioscience, the company that can turn a field of rice into a factory for producing human medicine.

There's the way Ventria sees itself -- as a biotechnology company hard at work on medical products that could save lives.

"Without our technology those products could never end up in use for human health, not in our lifetime," says Ventria Chief Executive Scott Deeter.

And then there's the view of opponents, who see the firm as a harbinger of a new biotech age that threatens the purity of the food supply and puts growers of conventional crops at risk.

"As a California rice farmer, I say: Don't grow drugs in my food crop," says Greg Massa, who grows rice in Glenn and Colusa counties.

Whichever view is correct, this tiny, 11-year-old Sacramento company is now front and center in a national debate over using food crops to grow drugs, as well as industrial and nutritional products.

Scores of companies and academic labs have been attempting this for years, confining their crops to greenhouses or to small field parcels.

Meanwhile, Ventria has been moving beyond the more typical small patches.

This year, Ventria hoped to grow up to 120 acres of its genetically engineered rice. But it was willing to limit production to one out of 10 selected California counties -- far enough south of the Central Valley rice belt to allay the concerns of growers who feared field contamination. But the California Secretary of Food and Agriculture blocked its permit, saying that he wants more time to hear from the public.

Deeter now says Ventria will likely plant only a small test field in California this year and may leave the state to plant a larger field in 2005.

Still, the mood remains decidedly upbeat at Ventria's headquarters in Sacramento.

Deeter says the privately held company, which has 20 employees, remains a few years away from commercializing its first products. It has spent more than $20 million on two human proteins that are usually found in mother's milk, tears and saliva but, through genetic manipulation, can also be produced in rice.

Sponsoring research The company is sponsoring research to show that in the right doses, the two proteins -- lactoferrin and lysozyme -- can be helpful in treating diarrhea.

Worldwide, as many as 3 million children under 5 die each year from the dehydration that accompanies severe diarrhea. Even in the United States, hundreds of youngsters and thousands of elderly patients die from it each year.

For decades, standard treatment has been a solution of table salt and carbohydrates. This summer, a clinic in Lima, Peru, will begin a Ventria-sponsored study to see if adding the lactoferrin and lysozyme, extracted from a flour made from the modified rice, gets even better results.

"The benefits of this approach are enormous," said Dr. William B. Greenough III, a professor of medicine at Johns Hopkins University.

Greenough was part of a team in Bangladesh that developed the standard treatment that is credited with saving the lives of 3 million infants each year.

Maryland company Greenough is also co-founder of Cera Products, a Maryland company that has been talking about adding Ventria's lactoferrin and lysozyme to its product for treating diarrhea.

Ventria is conducting studies in Los Angeles to see if lactoferrin, which plays a role in absorption of iron in the intestines, can help women suffering from anemia.

The company's critics don't dispute the potential benefits.

Instead, they see Ventria as opening a door to an industry that is still not adequately regulated.

For example, because genetically engineered lactoferrin and lysozyme are virtually the same as natural human proteins, the company can market them as "medical foods," a designation that does not require a detailed Food and Drug Administration review. But for a variety of reasons, Ventria is taking a more cautious approach and will ask the FDA to review the proteins for safety.

Environmentalists, rice growers and some experts worry about inadvertent mixing of Ventria's rice with the non-engineered food crop.

Voicing concerns "I have concerns that any pharmaceutical product grown in a food crop could end up in food," said Steve L. Taylor, professor of food science at University of Nebraska.

Even if the products prove safe, he said, "a lot of people have trouble thinking of Rice Krispies with human genes in them." Says Bill Reese, a research analyst with Friends of the Earth: "First of all, these are not human proteins." Small differences might trigger allergies and other unexpected responses, he said.

Even if there should be a mix-up between Ventria's rice and non-engineered food crops, the two human proteins pose no hazard to consumers, says Delia Bethell, Ventria's vice president

of clinical development. "If you breast-feed a baby for a year, that child consumes 277 grams of lactoferrin," she said. A person who ate Ventria's genetically modified rice over an entire year, she said, would consume only 60 grams.

VENTRIA BIOSCIENCE

The privately held company is genetically modifying rice plants to produce potentially useful proteins, turning fields of rice into factories for drugs, food supplements and other products.

Headquarters: Sacramento

Number of Employees: 20

Primary Products: Genetically engineered human lactoferrin and lysozyme for treating diarrhea and iron deficiency

Chief Executive Officer: Scott E. Deeter

Board Members: William J. Rutter and Pablo Valenzuela, co-founders of Chiron

The Benefits: Five thousand acres of rice can produce enough genetically engineered proteins to treat millions of diarrhea cases -- at less than 1 percent of the cost of making them in standard biotechnology factories.

Critics Say: Company's plans may threaten purity of the food supply and could put conventional growers at risk.

Rice Expressing Lactoferrin and Lysozyme Has Antibiotic-Like Properties When Fed to Chicks[1]

Brooke D. Humphrey, Ning Huang* and Kirk C. Klasing[2]

*Department of Animal Science, University of California, Davis, CA and *Ventria Bioscience, Incorporated, Sacramento, CA*

ABSTRACT Two experiments were conducted to determine whether rice that has been genetically produced to express human lactoferrin (LF) or lysozyme (LZ) protects the intestinal tract similarly to subtherapeutic antibiotics (bacitracin + roxarsone; Antibiotics). Experiment 1 compared 10 corn-soy diets containing 20% of various proportions of LF, LZ or conventional rice (CONV). Chicks fed 5% LF + 10% LZ + 5% CONV had significantly better feed efficiency and thinner lamina propria in the duodenum than those fed 20% CONV. Experiment 2 compared five corn-soy diets containing experimental rice combinations totaling 15% rice. Chicks fed 10% LZ + 5% CONV or 5% LF + 10% LZ had significantly lower feed intake and significantly better feed efficiency than those fed 15% CONV. Chicks fed 10% LZ + 5% CONV, 5% LF + 10% LZ or Antibiotics had significantly greater villous height in the duodenum compared with chicks fed 15% CONV. The lamina propria of the ileum was thinner and contained fewer leukocytes in chicks fed 10% LZ + 5% CONV or Antibiotics compared with those fed 15% CONV. The results from these experiments demonstrate a potential of genetically produced LF and LZ rice to be used as a substitute for antibiotics in broiler diets. J. Nutr. 132: 1214–1218, 2002.

KEY WORDS: • *antibiotics* • *lactoferrin* • *lysozyme* • *broilers* • *growth* • *intestinal morphology*

It is common practice to supplement the diets of poultry and pigs with antibiotics to improve their health, productivity and meat quality. However, the use of subtherapeutic antibiotics in animals negatively affects human health (1) due to the emergence in food animals of zoonotic microorganisms that are resistant to antibiotics (2,3). This may result in a decrease in the therapeutic effectiveness of antibiotics used to treat a variety of bacterial infections in humans. This threat to human health has prompted several European countries to ban their use, and in the United States, alternatives to antibiotics are currently being encouraged (3).

One strategy for replacing antibiotics in animal diets is to employ antibacterial molecules normally found along the digestive tract. Lactoferrin and lysozyme are present in mucosal secretions and in milk where they provide defense against bacteria along epithelial surfaces (4–6). Both of these molecules are highly resistant to hydrolysis by acids and proteases and to digestion in the gastrointestinal tract (7–10).

Lysozyme is a 1,4-β-acetylmuramidase that hydrolyzes the glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine (4). Hydrolysis products include murimyl dipeptide, a potent adjuvant capable of enhancing immunoglobulin A (IgA)[3] secretion, macrophage activation and rapid clearance of bacterial pathogens in vivo (11). Lysozyme is also capable of binding to the lipid A portion of bacterial endotoxin (12). Lysozyme-lipid A binding results in a conformational change that keeps endotoxin from interacting with macrophage receptors and dampens the release of the proinflammatory cytokines interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (13–15).

Lactoferrin is a cationic protein that has bacteriostatic and bactericidal effects that contribute to both systemic and mucosal immune defense. (16) Lactoferrin is a scavenger of free iron and acts to deprive microorganisms of the essential nutrient (17). Bactericidal activity stems from its ability to destabilize the outer membrane of gram-negative bacteria through the liberation of lipopolysaccharides (LPS) from their cell walls (18). Lactoferrin is also capable of reducing LPS-induced proinflammatory cytokine release by monocytes, as well as blocking the LPS priming of neutrophils for superoxide production (19).

Lysozyme and lactoferrin are efficacious for resistance to infectious diseases in experimental animals and humans after administration by oral, intravenous, intraperitoneal and topical routes. For example, intraperitoneal administration decreases the pathology resulting from a *Klebsiella pneumoniae* infection in mice (20). In rainbow trout, lysozyme injections decrease mortality from a challenge with *Aeromonas salmonicida* by more than threefold (21) and oral administration decreased mortality from infectious pancreatic necrosis virus by twofold (21). Oral administration of egg white lysozyme is now used clinically in human medicine for the therapy of inflammatory diseases of respiratory and digestive epithelia (22).

[1] Supported by University of California Biostar program grant #S99-24.
[2] To whom correspondence should be addressed.
E-mail: kcklasing@ucdavis.edu.
[3] Abbreviations used: Antibiotics (bacitracin + roxarsone), CONV, conventional rice; Ig, immunoglobulin; IL, interleukin; LF, rice genetically produced to express lactoferrin, LPS, lipopolysaccharides; LZ, rice genetically produced to express lysozyme, TNF, tumor necrosis factor.

Manuscript received 13 November 2001. Initial review completed 21 December 2001. Revision accepted 7 March 2002.

Antibiotics enable their growth-promoting effects, at least in part, by reducing stress caused by microbial challenges as indicated by lowered plasma IL-1 and corticosteroid levels (23). Therefore, other strategies that reduce bacterial challenges or decrease proinflammatory cytokines might be efficacious. The purpose of these experiments was to determine the efficacy of rice genetically produced to express human lactoferrin (LF) or lysozyme (LZ) to serve as a substitute for antibiotics in poultry diets. Expression of recombinant proteins in grains such as rice has the advantage over other systems, e.g., *Aspergillus*, *Saccharomyces* or tobacco, of eliminating the need to purify the transgenic protein from the producing organism before feeding

MATERIALS AND METHODS

Birds and management. Male Cobb broiler chicks (1 d old; Foster Farms, Delhi, CA) were raised in Petersime brooder batteries (Petersime Incubator, Gettysburg, OH) located in an environmentally controlled room (25°C) with 24 h light. Chicks were provided water and commercial chick starter for ad libitum consumption. The batteries had not been cleaned after their previous use to provide a level of sanitation conducive to an antibiotic response. When the chicks were 3 d old, experimental chicks were selected for uniform body weight from a twofold larger population and randomly assigned to dietary treatments. Chicks had ad libitum access to both feed and water and were exposed to a 24-h light cycle. All experiments and procedures were approved by the Campus Animal Care and Use Committee.

Rice. Two transgenic rice strains were produced to express either lactoferrin or lysozyme (24). Briefly, rice callus from the rice strain Taipei 309 was transformed with plasmids carrying genes for LF and LZ under the control of rice glutelin 1 gene promoter. Transgenic plants were screened for a high level of expression of both recombinant proteins. The selected lines, 159–53 and 164–12, were propagated to produce sufficient amount of rice seed for these experiments. LF and LZ rice expressed 4.0 and 2.5 g/kg of recombinant protein as determined by ELISA. Taipei 309, which is the conventional rice (CONV) that served as the host for transgenic plant production, served as a control All rice was dehusked to yield brown rice and then ground using a comminuting machine (Fitzpatrick, Chicago, IL).

Diets. Corn-soy-rice basal diets were formulated to meet or exceed the nutrient needs of young growing broiler chicks suggested by the NRC (25). All experimental diets were formulated to contain the same amount of rice by substituting transgenic rice for CONV rice (Tables 1 and 2). A range of levels of each test rice was chosen for study to determine a minimally efficacious level. The 10.0% LZ diet used in Experiment 1 was analyzed to contain 176 mg/kg lysozyme. After 6 mo of storage at room temperature, it contained 152 mg/kg lysozyme, indicating that this protein was stable to storage.

Experiment 1. In this experiment, 300 3-d old chicks were randomly assigned to 1 of 10 dietary treatments (Table 2). Each dietary treatment consisted of six replicates, with five chicks per replicate. Chick and feeder weights were determined on d 1 and 17.

Experiment 2. A second experiment was designed to confirm the results of the first experiment using twice the number of replicates per treatment to examine more subtle effects of treatments; 360 3-d old chicks were randomly assigned to one of 5 dietary treatments (Table 2) with 12 replicates per treatment and 6 chicks per replicate (42 chicks per treatment). Chick and feeder weights were determined on d 1 and 19.

Histology. For both Experiments 1 and 2, intestinal samples were taken on the last day of the experiment. Sections (2.5 cm) from one bird per replicate were obtained from the duodenum at the apex of the pancreas, the jejunum at a position midway between Meckel's diverticulum and the entrance of the bile ducts, the ileum at a position midway between Meckel's diverticulum and the ileum-cecal junction, and the ceca at a point midway along its length (Experiment 2 only). Samples were flushed with saline, fixed in 10% buffered formalin (pH 7.0), embedded with paraffin, thin-sectioned and stained with hematoxylin-eosin (IDEXX Veterinary Services, Sacramento, CA). For enumeration of intraepithelial and lamina propria leukocytes, sections were fixed in acetone, redried, incubated with mouse anti-chicken CD45 monoclonal antibody (Southern Biochemical Associates, Birmingham, AL) for 1 h and then rinsed in PBS Sections were incubated with rabbit anti-mouse Ig tagged with peroxidase with 5 g/L bovine serum albumin for 1 h and rinsed Peroxidase activity was developed by incubating sections with 0.01% H_2O_2 and 3,3′-diamino-benzidine-tetrahydrachloride. The slides were counterstained with hematoxylin-eosin. The number of leukocytes in 10 villi per section and the number of leukocytes in the lamina propria underneath and within these 10 villi were enumerated. Cells with endogenous peroxidase activity (primarily heterophils) were also enumerated as described by Vervelde and Jeurissen (26). For each intestinal sample, villi height, villi width, crypt depth, lamina propria thickness, number of lamina propria leukocytes and number of intraepithelial leukocytes were estimated using Image-Pro-Plus software (Media Cybernetics, Silver Spring, MD).

Statistical analysis. For both Experiments 1 and 2, data were analyzed for main effect of diet using the general linear model (Minitab; State College, PA). When main effects were significant ($P < 0.05$), differences due to dietary treatment were determined using Tukey's means comparisons.

TABLE 1

Composition of chick diets from Experiments 1 and 2

	Experiment 1 Corn-soy-rice	Experiment 2 Corn-soy-rice	Experiment 2 Corn-soy
		g/kg	
Ingredient			
Corn	354	408	551
Soy meal (48.5% protein)	320	343	342
Rice[1]	200	150	—
Poultry grease	57.9	43.1	51.0
Meat with bone meal	40.5	—	—
Feather meal	—	21.2	21.2
Calcium carbonate	—	7.6	8.0
Tricalcium phosphate	—	19.1	18.7
Calcium phosphate	18.5	—	—
Vitamin-mineral premix[2]	1.00	1.00	1.00
Choline	0.70	0.84	0.91
Sodium chloride	4.03	2.21	2.04
DL Methionine	2.78	2.03	1.97
L-Lysine	—	—	0.14
Calculated composition			
ME, *kJ/kg*	765	765	765
Crude protein, %	22.15	22.29	22.29
Crude fat, %	8.07	6.69	7.85
Available Lys, %	1.20	1.15	1.15
Available Met + Cys, %	0.95	0.88	0.88

[1] Three types of rice (conventional rice or rice expressing lactoferrin or lysozyme) at variable levels depending upon the treatment.

[2] Vitamins and minerals were provided in the form and level described in NRC (25) Standard Reference Diet for Chicks.

[3] Corn-soy diet for Experiment 2 only.

RESULTS

Experiment 1. Chicks fed diets containing 0.1% LF, 1% LF, 5% LF, human lactoferrin, 0.2% LZ, 10% LZ, or 0.1% LF + 0.2% LZ did not differ from chicks fed CONV in any of the parameters measured, and therefore will not be mentioned further. Feed intake and body weight gain were not affected by dietary treatments ($P > 0.05$), and averaged 36.10 and 28.96 g/(chick · d), respectively. Chicks fed 5% LF + 10% LZ had significantly greater feed conversion compared with chicks fed CONV (Table 3). Chicks fed antibiotics (bacitracin + rox-

TABLE 2

Types of rice in dietary treatments used in Experiments 1 and 2[1]

Dietary treatment	Conventional rice	Transgenic rice
	g/100 g diet	
Experiment 1		
CONV	20	0
0.1% LF	19.9	0.1
1.0% LF	19.0	1.0
5.0% LF	15.0	5.0
Human lactoferrin[2]	20	0
0.2% LZ	19.8	0.2
10.0% LZ	10.0	10.0
0.1% LF + 0.2% LZ	19.7	0.3
5.0% LF + 10% LZ	5.0	15.0
Antibiotics[3]	20	0
Experiment 2		
Corn-soy	0	0
CONV	15	0
10% LZ	5.0	10.0
5.0% LF + 10% LZ	0	15.0
Antibiotics[3]	15	0

[1] Conventional and transgenic rice totaled 20 and 15% of the diet for Experiment 1 and Experiment 2, respectively. The remainder of the diet is described in Table 1.

[2] Incorporated at 0.2 g/kg diet.

[3] Antibiotic treatments contained roxarsone (3-nitro-4-hydroxyphenylarsonic acid; Pfizer, Inc. New York, NY) and bacitracin methylene disalicylate (Schering-Plough Animal Health, Omaha, NE) at 0.25 diet and 0.5 g/kg diet, respectively.

arsone; Antibiotics) also tended ($P = 0.058$) to have greater feed conversion than those fed CONV.

Histological characteristics of the duodenum, jejunum and ileum are presented in **Table 4**. There were no significant differences in villous height, villous width or crypt depth due to dietary treatments in any intestinal segment (data not shown). Chicks fed 5% LF + 10% LZ had significantly thinner lamina propria in the duodenum compared with those fed CONV ($P < 0.05$). Chicks fed Antibiotics had jejuni with significantly thinner lamina propria and lower counts of lamina propria leukocytes compared with chicks fed CONV ($P < 0.05$). Chicks fed 5% LF + 10% LZ or Antibiotics tended ($P = 0.068$) to have lower counts of lamina propria leukocytes in the ileum compared with those fed CONV.

TABLE 3

Feed efficiency of chicks fed modified rice expressing lactoferrin (LF) and lysozyme (LZ) compared with those fed conventional rice (CONV) or subtherapeutic antibiotics (Experiment 1)[1]

	Feed efficiency
	g body weight gain/g feed consumed
Dietary treatment	
CONV	0.79 ± 0.1^{a}
5% LF + 10% LZ	0.84 ± 0.1^{b}
Antibiotics	0.82 ± 0.1^{ab}

[1] Values are means ± SEM, $n = 6$. Means not sharing a letter differ, $P < 0.05$.

TABLE 4

Intestine histology of chicks fed modified rice expressing lactoferrin (LF) and lysozyme (LZ) compared with those fed conventional rice (CONV) or subtherapeutic antibiotics (Experiment 1)

	Lamina propria thickness	Intraepithelial leukocytes	Lamina propria leukocytes
	μm	*n/villi*	
Duodenum:			
CONV	101^{b}	4.8	34.3
5% LF + 10% LZ	82^{a}	4.0	24.5
Antibiotics	85^{ab}	4.75	25.5
SEM[1]	3.06	0.73	3.35
P-value	0.015	0.543	0.196
Jejunum:			
CONV	101^{b}	8.0	38^{b}
5% LF + 10% LZ	97^{ab}	5.0	21^{ab}
Antibiotics	84^{a}	2.5	14^{a}
SEM[1]	3.26	1.06	3.67
P-value	0.011	0.061	0.008
Ileum:			
CONV	105	0.38	34
5% LF + 10% LZ	86	0.34	17
Antibiotics	83	0.39	18
SEM[1]	6.5	0.03	3.67
P-value	0.111	0.494	0.068

[1] Values are means, $n = 6$. Means in a column within intestinal segment not sharing a superscript differ, $P < 0.05$.

Experiment 2. Chicks fed the corn-soy diet, which was devoid of rice, did not differ from chicks fed CONV in any of the parameters measured, and therefore will not be mentioned further. There was no significant difference ($P > 0.05$) in body weight gain due to dietary treatments, which averaged 37.89 g/(chick · d). Chicks fed 5% LF + 10% LZ or 10% LZ consumed significantly less feed than those fed CONV (**Table 5**). Chicks fed either 10% LZ, 5% LF + 10% LZ, or Antibiotics had greater feed efficiency compared with chicks fed CONV (Table 5). As in the first experiment, there were no differences in body weight gain, food intake or feed efficiency between chicks fed LF or LZ rice and those fed Antibiotics.

Chicks fed 10% LZ, 5% LF + 10% LZ or Antibiotics had significantly greater villous height in the duodenum than

TABLE 5

Food intake and feed efficiency of chicks fed modified rice expressing lactoferrin (LF) and lysozyme (LZ) compared with those fed conventional rice (CONV) or subtherapeutic antibiotics (Experiment 2)[1]

	Food intake	Feed efficiency
	g/(chick · d)	*g body weight gain/ g feed consumed*
Dietary treatment		
CONV	52.18 ± 1.09^{b}	0.72 ± 0.01^{a}
10% LZ	48.05 ± 1.11^{a}	0.77 ± 0.01^{b}
5% LF + 10% LZ	49.02 ± 1.23^{a}	0.77 ± 0.01^{b}
Antibiotic	50.48 ± 1.15^{ab}	0.75 ± 0.01^{b}

[1] Values are means ± SEM, $n = 12$. Means in a column not sharing a common superscript differ, $P < 0.05$.

TABLE 6

Intestine histology of chicks fed modified rice expressing lactoferrin (LF) and lysozyme (LZ) compared with those fed conventional rice (CONV) or subtherapeutic antibiotics (Experiment 2)

	Lamina propria thickness	Villous height	Lamina propria leukocytes
	μm		*n/villi*
Duodenum			
CONV	81	743[a]	24
10% LZ	77	884[b]	21.5
5% LF + 10% LZ	82	882[b]	23.3
Antibiotics	81	876[b]	19.2
SEM[1]	2.46	18.3	2.1
P-value	0.69	0.016	0.93
Ileum			
CONV	99[b]	365	28.3[b]
10% LZ	69[a]	405	17.8[a]
5% LF + 10% LZ	88[ab]	421	25.2[ab]
Antibiotic	75[a]	356	18.7[a]
SEM[1]	3.2	12.6	1.5
P-value	0.03	0.36	0.02

[1] Values are means, $n = 12$. Means in a column not sharing a common superscripts differ, $P < 0.05$.

chicks fed CONV (Table 6). Chicks fed CS + 10% LZ or Antibiotics had significantly thinner lamina propria in the ileum and fewer leukocytes in the ileal lamina propria compared with chicks fed CONV. There were no other significant differences due to diet in villous width, crypt depth or intraepithelial leukocytes in any intestinal segment.

DISCUSSION

The purpose of these experiments was to determine the potential of rice expressing either lactoferrin or lysozyme transgenes to serve as an antibiotic replacement in broiler diets as indicated by improved performance characteristics and intestinal morphology. Lactoferrin, lysozyme and IgA are the primary protective factors in epithelial secretions and in milk. Milk lysozyme and lactoferrin concentrations are highly dependent upon species and stage of lactation. Human milk contains 1.9 g/L lactoferrin at 1 mo of lactation diminishing to 1 g/L at 1 y. Lysozyme is present at 0.02 and 0.2 g/L at 1 mo and 1 y, respectively (27). Our highest level of LZ (10% of the diet) provided 0.3 g/kg lysozyme, which is similar to the highest concentrations in human milk. Inclusion of LF at 5% provided 0.125 g/kg, which is low relative to human milk. We were precluded from testing higher levels of lactoferrin because of scarcity of this strain of plant at the time of these studies.

Previous investigators demonstrated that the inclusion of antibiotics in the diet results in increased growth rate and improved feed efficiency compared with controls fed antibiotic-free diets (23,28–32). In our experiments, feed efficiency tended to be improved by dietary antibiotics in the first experiment and was significantly improved in the second experiment, in which a greater number of replicates were used. We did not observe an effect of dietary antibiotics on weight gain and this could be due to lower efficacy of antibiotics in a clean environment (23,33,34). Our experiments were conducted in cages, whereas much of the published work has utilized floor pens containing reused litter. Although sanitation standards were relaxed in our cage environment, cleanliness was still better than what is typically found in reused litter environments. Diets containing 5% LF + 10% LZ improved feed efficiency in both experiments and diets containing 10% LZ resulted in an improvement in the second experiment. Diets containing the highest levels of LF and LZ were at least as effective in improving feed efficiency as antibiotics.

Antibiotics are thought to improve animal productivity by lowering microbial infectious challenges along the gastrointestinal tract (23,35). This mode of action is supported by the observation that antibiotics are ineffective at improving animal productivity in a germfree environment (23,31,34). Antibiotic-fed chicks have lower small intestinal weights than chicks fed antibiotic-free diets (36–38). The difference in intestinal weight is not due to alterations in fat, moisture content or length, (36) but rather to changes in intestinal tissue layers (33,37,39). Jukes et al. (37) demonstrated that antibiotics caused significantly thinner duodenal lamina propria and cross-sectional diameter. Small intestines of chicks raised in a germfree environment also have diminished lamina propria and a greater proportion of epithelial layer compared with conventionally raised chicks (39). We observed reduced thickness of the lamina propria and increased villi length in the small intestine of chicks fed either antibiotics or high levels of LZ and LF. Presumably the increased villi length, and consequently surface area, would facilitate better nutrient digestion and absorption, explaining the improved efficiency of feed utilization.

Both germfree and antibiotic-fed chicks have lower counts of reticuloendothelial cells in the subepithelial tissue of their small intestines and lower ileocecal tonsil weights compared with conventionally raised or antibiotic-free chicks, respectively (31,40,41). In our second experiment, we found that the diminished number of leukocytes in the lamina propria of the small intestine due to antibiotics was duplicated by 10% LZ. Fewer leukocytes suggest lower levels of microbial challenges and improved intestinal health due to these dietary treatments.

We observed effects of antibiotics and rice on the thickness of the lamina propria and numbers of lamina propria leukocytes in both experiments, yet the region of the small intestine in which they occurred differed. This variability might be explained by the difference in the age at which the birds were sampled. Alternately, the differences could be due to the microbial populations that colonized the intestines. The microbial ecology of the intestines is extremely variable over time and it is almost certain that there were differences in the communities of microbes inhabiting the intestines of the birds used in these two experiments. These differences could easily explain the variability in the intestinal regions in which we observed treatment effects.

In general, we found that the combination of 5% LF + 10% LZ was more efficacious at improving feed efficiency and histological indices of intestinal health than 10% LZ alone; 5% LF alone was without effect. These observations suggest that at the levels used, lysozyme was much more efficacious than lactoferrin and that there was synergism between these two proteins. In vitro studies utilizing a combination of lactoferrin and lysozyme on bacterial growth have demonstrated increased bacteriostatic activity compared with either lactoferrin or lysozyme alone (5). Lactoferrin binds to the lipid A component of LPS of several serotypes of bacteria involved in septic shock, resulting in the release of LPS from the membrane and enhanced susceptibility of the bacteria to lysis by lysozyme (5,16).

In vitro, lactoferrin decreases the production of IL-1 and TNF-α from mixed lymphocyte cultures (19). Lysozyme de-

creases in vitro IL-6 production by macrophages, and in vivo production of TNF-α (13,14). The proinflammatory cytokines IL-1, IL-6 and TNF-α mediate the impaired growth rates and feed efficiency that accompany the immune response to bacteria and their LPS (42,43). Roura et al. (23) demonstrated that feeding antibiotics lowers circulating bioactive IL-1 levels, which presumably permits faster growth and better feed efficiency. Whether the effects of LF and LZ were mediated by the bacteriostatic properties of lactoferrin and lysozyme, by their modulation of pro-inflammatory cytokine profiles or by a combination of both remains to be determined.

Feeding antibiotics remains efficacious at improving animal productivity after > 50 y of steady use. However, evidence suggesting the transfer of antibiotic resistance genes from commensal bacteria of poultry, pigs and cattle to human pathogens has driven a search for alternate strategies. Our experiments demonstrate the potential of rice expressing lactoferrin and lysozyme to serve as an alternative to antibiotics in broiler diets. The antibacterial properties of lysozyme and lactoferrin evolved in the presence of commensal and pathogenic microflora, and there is no indication that bacteria have become resistant to these proteins in nature. However, the development of bacterial resistance after prolonged feeding of these proteins has yet to be examined.

The approach of reinforcing the innate immune system by feeding protective proteins normally found along the intestinal epithelium may have applications beyond chickens. In particular, weanling baby pigs are very reliant on dietary antibiotics for maintenance of intestinal health and prevention of diarrhea. Because the transgenic proteins used in these experiments were of human origin, this strategy may also be relevant to the nutrition of human infants.

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Agennix Incorporated

December 20, 2005

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Re: RhLF GRAS Meeting Follow-up

Dear Dr. Tarantino:

Thank you for meeting with us on November 14th regarding safety issues raised by GRAS Notice No. 000162 for recombinant human lactoferrin (rhLF) produced in rice.

We were glad to have the opportunity to discuss our concerns with you, and would like to be responsive to questions asked during and following our meeting. Dr. Luccioli and Dr. Mattia had inquired, following our meeting, about our understanding of rhLF's mechanism of action. Agennix has conducted substantial research that we believe further elucidates rhLF's mechanism of action and better explains why orally administered rhLF has such a complex and potent effect on immune response. We have attached a brief confidential presentation that outlines our research results in this area (**APPENDIX 1**).

Second, I believe you inquired during the meeting about the significance of the risks relating to foreign glycosylation on rhLF, considering that we regularly consume plants with similar glycosylation. The concerns articulated by Dr. Cummings on this issue included the fact that rhLF could act to deliver a high concentration of known allergenic carbohydrates in a single bolus, while in a regular diet these carbohydrates are diluted and masked by other plant components and inert material such as cellulose. Of potentially greater concern is the fact that rhLF itself acts as a targeted delivery device. As described in our confidential mechanistic package, rhLF binds the gut mucosa, makes its way into the Peyer's Patches and induces chemotaxis of antigen presenting cells (APCs) and immune effector cells to the Peyer's Patches. Thus, rhLF facilitates the

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delivery of foreign carbohydrate antigens directly to APCs including dendritic cells and macrophages.

Third, we received input from an additional expert, John Axford, M.D. (St George's hospital, London), who was not represented in our scientific assessment. We had contacted Dr. Axford prior to our meeting, but because of his travel schedule his views could not be included in the scientific assessment in time for its submission prior to our meeting. Dr. Axford is an expert in rheumatology and expressed strong concerns about rhLF's potential long-term effects on autoimmunity. Since autoimmunity is an important safety issue, we thought that you would find Dr. Axford's comments, as reflected in his attached letter (**APPENDIX 2**), helpful to your evaluation.

Fourth, Dr. Merker recently requested references providing evidence of allelic variation (polymorphisms) in the human lactoferrin gene. This subject was briefly addressed and referenced on page 7 of the scientific assessment submitted prior to our meeting. Numerous additional studies have documented lactoferrin gene polymorphisms. To provide you with further documentation, a representative set of additional references has been attached in **APPENDIX 3**.

Fifth, in his statements at our meeting, Dr. Cummings (one of the experts) commented on two papers, and we would like to provide you with these references. In the first paper, which was recently published in Nature (Ju and Cummings 2005), Dr. Cummings found that a genetic mutation occurring spontaneously in hematopoietic stem cells of patients with Tn Syndrome is responsible for expression of an altered carbohydrate called the Tn antigen. The Tn antigen was found to be strongly antigenic. This work illustrates how even minor alterations in carbohydrate structures can have tremendous immune consequences, and suggests that caution is required when administering recombinant proteins with altered glysosylation to people.

Dr. Cummings also made reference to a second paper (Gomord 2004) and read a passage from page 92 that states: "As a consequence, for safety concerns related to the use of plant-derived therapeutic proteins, further experiments should be carried out in an appropriate animal model, as well as in human volunteers, by administering a therapeutic glycoprotein produced in a plant and analyzing the immune responses to the plant glyco-epitopes in allergic and non-allergic populations." Dr. Cummings's comments underscored the need for rigorous clinical and preclinical testing to determine the safety of therapeutic glycoproteins produced in plants. Both of these papers are provided in **APPENDIX 4**.

Finally, we would like to bring to your attention an additional paper that was just recently published (Prescott 2005). The paper, which is also attached for your reference in **APPENDIX 5**, described a study that demonstrated that expression of a recombinant protein in a non-native host resulted in an altered protein and increased immunogenicity. Specfically, the expression of one plant protein (bean α-amylase inhibitor; αAI) in a non-native plant species (peas) resulted in post-translational protein modifications (including variations in glycosylation). Although oral consumption of the native bean form of αAI did not promote immunological responsiveness or inflammation in mice, oral consumption of the transgenic αAI did induce αAI-specific systemic immunological responsiveness as measured by a variety of parameters including IgG titers, DTH responses, and TH2-type pulmonary inflammation. Furthermore, co-administration of the transgenic αAI with other dietary proteins promoted immunological cross priming and elicited immunogencity to unrelated dietary proteins. The study demonstrates the structural and immunological consequences that can result from transgenic expression of proteins even in relatively closely related species such as two plants.

We believe the additional information above reinforces and even strengthens our position taken at our November 14th meeting, which is that GRN No. 00162, as submitted by Ventria Bioscience for rice-derived recombinant human lactoferrin, does not meet the statutory criteria for being found Generally Recognized as Safe (GRAS). We believe that GRN 000162 fails on all three parts of the GRAS standard:

1. That it fails to establish technical evidence of safety. In particular, the differences in glycosylation between the Ventria product and both native human lactoferrin and other recombinant human lactoferrins are significant and raise important safety questions concerning the potential for allergenicity and immunogenicity, among others.

2. That it fails to provide adequate public information on which to base a GRAS determination, as most of the data in GRN 000162 are either unpublished or are on a different compound (i.e., the Agennix data that is not directly relevant).

3. That it fails to establish a general recognition among qualified experts. Indeed, with the added submission of the attached letter from John Axford, FDA now has the views of EIGHT leading experts in the field who all believe that significant safety concerns remain with GRN 000162. This clearly demonstrates that there is a "severe conflict" within the scientific community on this subject.

Accordingly, we believe Ventria's submission on rhLF should be denied.

Thank you and your staff again for your diligence and attention to this important issue. Please do not hesitate to contact me again if additional information or clarification is needed.

Sincerely yours,

Richard Barsky

Richard Barsky
Chief Executive Officer
Agennix, Inc.

Cc: Robert I. Merker, Ph.D. (HFS-255)
Consumer Safety Officer
Division of Biotechnology and GRAS Notice Review

Frank E. Young, M.D., Ph.D.
Chairman of the Board
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Appendix 1

Agennix

A Biopharmaceutical Company Developing Drugs for Cancer and Diabetic Ulcers

Cancer Mechanism of Action

December 12, 2005

Pages 7-28 withheld under (b)(4)

Appendix 2

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The Sir Joseph Hotung Centre for Musculoskeletal Disorders

JSA/cc/2005/research/agennix

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29 November 2005

Dear Peter

Re: **Safety Concerns raised by Recombinant Human Lactoferrin Produced in Rice: Scientific Assessment of Gras Notice No. 000162 Submitted by Ventria Bioscience**

Thank you for letting me have sight of the above document and asking me for my comments.

I am a clinician with a specialist interest in rheumatology. My specific research interests concern glycobiology, where I have demonstrated that there are specific protein glycosylation changes associated with rheumatoid arthritis, that these changes may be associated with pathological mechanisms leading to inflammation and that these changes may be a useful diagnostic tool.

My specific concerns with regard to the information you have provided to me are that adequate research with regard to the glycosylation of rhLBF from rice has not been extensively investigated. This means that when ingested, an unusual glycosylation profile will be presented to the immune system and that the body may react as though this is a foreign antigen. Our work has demonstrated that immunoglobulin glycosylation in rheumatoid arthritis is defective in that the level of galactose is significantly reduced. We have demonstrated that this leads to increased anti-genicity of immunoglobulin G molecules and the likely consequence is that immune complexes are formed. In several studies it has been noted that reduced galactosylation is found within immune complexes in rheumatoid arthritis. Deposition of immune complexes is known to be associated with generation of inflammation and our published data therefore links aberrant glycosylation with an inflammatory process.

With regard to rhLF from rice, it is conceivable that an inflammatory mechanism could be associated if an aberrantly glycosylated molecule is ingested. In this respect, it is noted that anti-lactoferrin antibodies have been associated with a number of autoimmune diseases.

Cont/d.....

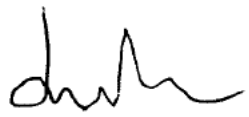
Professor John S. Axford DSc MD FRCP
Chair of Clinical Rheumatology, Director of the Hotung Centre

In addition to this, I would also concur with the points that were made in this document with regard to:

- Recombinant proteins are not identical to native analogues
- Bioactive active proteins should be viewed as drugs rather than food supplements
- The immunoregulatory affects of these molecules should be rigorously evaluated, especially, as changes in glycosylation profile may alter any expected affect.

I hope these comments are of use to you. Below I list relevant peer reviewed publications from which my comments are based.

Yours sincerely



John Axford

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Appendix 3

APPENDIX 3

Additional Documentation of the Allelic Variation of Lactoferrin in Humans

Polymorphisms of the lactoferrin gene have been documented in healthy individuals and in patients with diseases including cancer.

Furmanski et al, first described variants forms of lactoferrin which differed both functionally and in their antigenic properties. Lactoferrin gene polymorphisms have been detected within exons 2, 5, 7, 9, 13, 14 and 15 of normal placental and leukocyte DNA while abnormal migration patterns of the lactoferrin gene have been detected in cancer cells (Liu 2002). The current view is that genetic polymorphisms of the lactoferrin gene exist in selected exons and that additional mutations of the lactoferrin gene may also occur in cancer cells. Delta-lactoferrin (deltaLf) mRNA, which is the product of an alternative (P2) promoter present in the first intron of the lactoferrin gene, has been found in normal tissues (Breton 2004).

Genetic differences leading to the encoding of variant forms of lactoferrin protein may result in altered biologic activity and function. For example, genotypic analysis of a small population of localized juvenile periodontitis patients and healthy subjects revealed a dramatic difference in the frequencies of the hLf-K and hLf-R alleles in these two groups. The hLf-K allele occurred much more frequently in localized juvenile periodontitis patients than in healthy subjects (Velliyagounder 2003). Similarly, a threonine/alanine substitution was associated with an increase in the risk of aggressive periodontitis with a P=0.0007 (Jordan, 2005). Conversely, the occurrence of specific lactoferrin variants may confer resistance to diseases such as infection by human herpesvirus-8 (Grange, 2005). Further, eight different types of nucleotide substitutions and one frameshift mutation of lactoferrin have been described, raising the possibility that the lactoferrin gene may be modified by genetic and epigenetic mechanisms (Iijima 2005).

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Appendix 4

PROTEIN GLYCOSYLATION

Chaperone mutation in Tn syndrome

Tn syndrome is a rare autoimmune disease in which subpopulations of blood cells in all lineages carry an incompletely glycosylated membrane glycoprotein, known as the Tn antigen. This truncated antigen has the sugar *N*-acetylgalactosamine α-linked to either a serine or threonine amino-acid residue[1,2], whereas the correct T antigen has an additional terminal galactose; the defect may be due to a malfunction of the glycosylating enzyme T-synthase[3,4]. Here we show that Tn syndrome is associated with a somatic mutation in *Cosmc*, a gene on the X chromosome that encodes a molecular 'chaperone' that is required for the proper folding and hence full activity of T-synthase[5]. The production of the autoimmune Tn antigen by a glycosyltransferase enzyme rendered defective by a disabled chaperone may have implications for other Tn-related disorders such as IgA nephropathy, a condition that can result in renal failure.

We used whole blood from two male donors with Tn syndrome (C.C. and C.L.) and from 25 healthy donors (male and female) with a total of 33 *Cosmc* alleles between them. T-synthase activity in whole-blood cell extracts from C.C. and C.L. was significantly lower (decreased by more than 60%) than that in control samples. Tn antigens, and Tn antigens carrying additional sialic acid sugar residues, were present on erythrocytes and leukocytes from C.C. and C.L., but not on blood cells from healthy donors.

The T-synthase gene (*T-syn*; chromosome position, 7p14–p13) contains three exons[3], whereas *Cosmc* has a single exon of 954 base pairs (chromosome position, Xq23)[5]. To determine whether the defective T-synthase activity in C.C. and C.L. might be correlated with mutations in these genes, we sequenced *Cosmc* and *T-syn* from whole blood cells. *T-syn* sequences were normal for all donors, but *Cosmc* sequences from C.C. and C.L. were mosaic, containing both normal and mutated sequences. *Cosmc* from C.C. has a substitution at nucleotide 202 that gives a premature stop codon instead of an arginine residue at position 68, and a polymorphism at nucleotide 393 that causes a conservative change from aspartate to glutamate at position 131; *Cosmc* from C.L. is mutated at nucleotide 454 to give lysine instead of glutamate at position 152 (Fig. 1a, and see supplementary information).

We found that the mutation at nucleotide 202 occurred in 6 of 14 *Cosmc* clones from C.C. and that the change at nucleotide 393 was present in all 14 of his clones; C.L.'s nucleotide 454 mutation occurred in 6 of 8 clones. As *Cosmc* is X-linked and the two donors are male, these *Cosmc* sequences must be mutated in only a subset of blood cells in both. Normal *Cosmc* sequences from the 25 healthy donors, representing 33 alleles, were identical[5]. The mutation in *Cosmc* found in the two donors with Tn syndrome is statistically significant ($P < 0.01$ in Fisher's exact test).

T-synthase activity relies on coexpression with Cosmc[5], so to test the effect of the *Cosmc* mutations on the chaperone's function, we expressed recombinant *Cosmc* (wild type and mutants) together with *T-syn* in the insect cell line known as Hi-5 (Fig. 1b). As expected, coexpression of *T-syn* with *Cosmc* from C.C. that had the conservative amino-acid substitution (thymine-to-adenine polymorphism at nucleotide 393) gave normal T-synthase activity. However, coexpression with C.C.'s truncated mutant Cosmc (cytosine changed to thymine at nucleotide 202) gave less than 10% of the T-synthase activity associated with wild-type Cosmc, and no activity was detectable with C.L.'s Cosmc mutant. Expression of recombinant *T-syn* in Hi-5 cells was equivalent in all cases.

These results indicate that the specific mutations in *Cosmc* from patients with Tn syndrome cause it to lose its chaperone function. We confirmed by western-blot analysis that Cosmc protein was normally expressed from complementary DNA encoding wild-type Cosmc or C.C.'s polymorphic Cosmc. By contrast, C.C.'s truncated 68-amino-acid Cosmc was not detected, although C.L.'s mutant Cosmc, which had no chaperone activity, was detected and was normal in size.

It has been suggested that Tn syndrome is clonal and somatic[6–8]. Our findings indicate that a somatic mutation in *Cosmc* in a subpopulation of multipotential haematopoietic stem cells in patients with Tn syndrome inhibits its chaperone activity and leads to inactivation of T-synthase and the expression of the autoimmune Tn antigen on blood cells of all lineages. This discovery may provide insight into the molecular basis for other Tn-related disorders, such as IgA nephropathy[9] and Henoch–Schönlein purpura[9], in which somatic mutations in *Cosmc* in haematopoietic precursors could contribute to disease aetiology.

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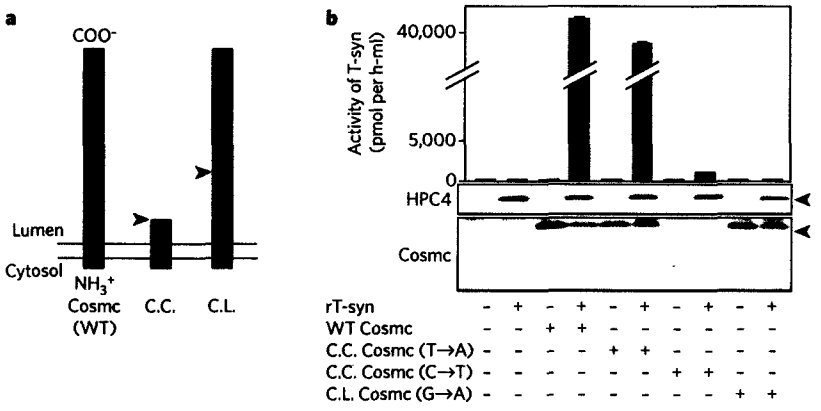

Figure 1 | Effect of *Cosmc* mutations on functional activity in patients with Tn syndrome. **a,** Length comparison of newly synthesized wild-type Cosmc, which is a protein of the endoplasmic reticulum that acts as a molecular chaperone for T-synthase (T-syn), with the mutated forms in patients C.C. and C.L. (arrows indicate mutation sites). **b,** Effect of coexpression of wild-type (WT) or mutant forms (C.C., +393T➛A or +202C➛T; C.L., +454G➛A, where notation numbering indicates the nucleotide mutation site and standard letter notation is used for the bases) of Cosmc on the activity of T-syn. Plasmids encoding these Cosmc variants were constructed and baculoviruses prepared in Sf-9 cells[5] (for methods, see supplementary information). Insect Hi-5 cells were infected with baculoviruses encoding human soluble HPC4-tagged recombinant T-syn and Cosmc, as indicated. Top, T-syn activity in cell medium was measured in triplicate (± s.e.m.); bottom, western blots of protein in cell medium using mouse anti-HPC4 monoclonal antibody (IgG1) to detect HPC4-tagged recombinant T-syn, and blots of protein in cell extracts using chicken anti-human Cosmc polyclonal antibody (IgY) to detect Cosmc. Migration positions of T-syn (M_r about 40K) and Cosmc (M_r about 37K) are indicated by arrowheads.

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REVIEW

Production and glycosylation of plant-made pharmaceuticals: the antibodies as a challenge

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Summary

Antibodies have long been recognized for their diagnostic and therapeutic potential. The rapidly increasing number of monoclonal antibodies approved for immunotherapy has paved the way to an even greater demand for these molecules. In order to satisfy this growing demand and to increase the production capacity, alternative systems based on antibody production in transgenic organisms are being actively explored. In this paper, we focus on transgenic plants as a promising system for the scale-up and processing of plant-made pharmaceuticals. In particular, we point out the advantages and limitations induced by glycosylation of plant-made antibodies for human therapy.

Introduction

For several decades, genetically modified organisms (GMOs) have been used to produce therapeutic molecules in a recombinant form. For a long time, the only GMOs used for such production were *Escherichia coli*, yeast and mammalian cells. Over the last 20 years, several proteins of pharmaceutical interest have also been produced in transgenic plants. These include blood and plasma proteins, such as human albumin (Sijmons *et al.*, 1990; Goddjin and Pen, 1995), aprotinin (Zhong *et al.*, 1999), enkephalin (Vandekerckhove *et al.*, 1989; Takamatsu *et al.*, 1990), haemoglobin (Dieryck *et al.*, 1995, 1997; Halling-Sorensen *et al.*, 1998), antigens produced for vaccination purposes (Richter *et al.*, 2000), growth factors, hormones, cytokines (Goddjin and Pen, 1995; Matsumoto *et al.*, 1995; James *et al.*, 2000), enzymes with therapeutic potential, conversion enzymes, angiotensin I (Hamamoto *et al.*, 1993), protein C (Cramer *et al.*, 1996), glucocerebrosidase (Cramer *et al.*, 1996; Steiner, 2000) and, finally, antibodies (Table 1, for illustration). Through this last example, we wish to illustrate the interest that molecular farming offers as an alternative to the current production systems of therapeutic molecules. We also wish to introduce the advantages and limitations associated with the glycosylation of plant-made pharmaceuticals (PMPs).

Plants as an alternative for the production of monoclonal antibodies

Antibody-based therapies (immunotherapies) have been highly developed over recent years. As early as 1998, 30% of the clinical trials concerning biotechnology-derived molecules involved antibodies (Hudson, 1998). This percentage has increased within the last 2 years, with almost 1000 therapeutic antibodies currently being tested for immunotherapies against various pathologies, such as immune system disorders, inflammatory diseases, cancers, disorders of the central nervous system and infectious diseases. More than 10 antibodies have already been clinically validated (Table 2), and more than 50 others may be administered clinically within the next 5 years. Immunotherapy often requires large quantities of antibodies. As an example, some treatments, such as the use of the Herceptin antibody (Herceptin) for breast cancer treatment, require the administration of 2–5 g of antibody per patient per year.

At the present time, most recombinant antibodies approved by the Food and Drug Administration (FDA) are produced in cultured Chinese hamster ovary (CHO) cells. This process is highly expensive and of limited capacity. Indeed, the investment needed to establish and operate a culture unit for mammalian cells is important (about 1 million $US for the

Table 1 Recombinant antibodies produced in transgenic plants (see Figure 1 for illustration of the different antibody and antibody fragments presented in the second column)

Antigen	Type of antibodies	Indications	Transformed plant	Promoter	Targeting signal	Expression	Reference
Immunomodulations							
Cutinase	scFv	ND	*N tabacum* cv Samsum		No SP	+	Schouten *et al* (1996, 1997)
	scFv		*S tuberosum*		No SP + KDEL	+ 0 2–1% of TSP	
Tobacco mosaic virus	IgG-2b	Reducing virus infectivity	*N tabacum* cv petit Havana	35 S promoter +	MSP	15–45 µg/g wet weight	Voss *et al* (1995) Fischer *et al* (1999)
A thaliana cyclin-dependent kinase	scFv	Immunomodulation in plant	Tobacco leaves	35 S promoter	No targeting signal	+ 1 2–1 8% of TSP	Eeckhout *et al* (2000)
Potato starch branching enzyme A	Llama VHH antibody fragment	Inhibition of enzyme function	*S tuberosum* (potato tubers)	35 S promoter	Chloroplast targeting peptide of granule-bound starch synthase	+ (0 03% of TSP)	Jobling *et al* (2003)
Environment							
Plant pathogenic Mollicute (bacteria)	scFv 2A10	Plantibody controlled resistance to mycoplasma infection	*N tabacum* cv PBD6	35 S promoter	Pectate lyase SP	+	Le Gall *et al* (1998)
Urea herbicide paraquat Herbicide atrazine	scAbs	Detection and removal of organic pesticides from contaminated water	*N tabacum* cv Xanthi	35 S promoter	Plant PR1a SP	+ 0 006–0 028% of TSP	Longstaff *et al* (1998)
Antibody-based therapy (human and animal health)							
Human carcinoembryonic antigen	Mouse/human chimeric IgG1 antibody (cT84 66)	Antibody-mediated cancer therapy (colon cancer, breast cancer and tumour with epithelial origin)	*N tabacum* cv petit Havana SR1 (transient expression)	35 S promoter	MSP	+ 1 mg/kg of FLW	Vaquero *et al* (1999)
	scFv T84 66				MSP + KDEL MSP	+ + 5 mg/kg of FLW	
	T84 66/G68 diabody		*N tabacum* cv petit Havana SR1	35 S promoter	Plant codon optimized SP	+ (1–5 mg/kg)	
					SP + KDEL	+ (4–12 mg/kg)	Vaquero *et al* (2002)
	scFvT84 66		*Triticum aestivum* L cv bobwhite *O sativa* L indica cv M12 and Bengal	Maize ubiquitin promoter or 35S promoter	MSP MSP + KDEL	+ wheat and rice 30–100 ng/g FLW + wheat 50–900 ng/g of FLW rice 1 5–29 µg/g of FLW	Stoger *et al* (2000)
Rabies virus protein	Monoclonal antibody (mAb SO57)	Rabies virus neutralization	*N tabacum* cv Xanthi	35 S promoter	MSP + KDEL	+ 3 µg/g of FLW (0 07% of TSP)	Ko *et al* (2003)

Human IgG monoclonal antibody	C5-1 IgG	Anti-human globulin reagent for phenotyping and cross-matching red blood cells of receivers and donors	*M sativa*	35 S promoter	MSP	+ 0 13–1 0% of TSP	Khoudi *et al* (1999)
Streptococcal surface antigen SAI/II	Guy's 13 IgG IgA/G sIgA/G	Tooth decay	*N tabacum*	35 S promoter	MSP	+ + 200–500 µg/g FLW (5–8% of TSP)	Ma *et al* (1994) Larrick *et al* (2001) Ma *et al* (1998) Ma *et al* (1995)
Colon cancer surface antigen	CO-17 A IgG	Antibody-mediated cancer therapy	*N benthamiana*		MSP MSP + KDEL		Verch *et al* (1998)
HSV-2, protein from herpes simplex virus (HSV)	IgG, IgA, DigA or sIgA IgG1 Fab and F(ab')$_2$	Immunoprotection against genital herpes and vaginal transmission of HSV	*O sativa* *G max*			+	Briggs *et al* (2000) Zeitlin *et al* (1998)
Zearalenone (mycotoxin)	scfv	Passive immunization of animals in their feed	*A thaliana* (ecotype Columbia)	Inducible *Lac* promoter	No SP Plant PR1-b SP	– +	Yuan *et al* (2000)
Human creatine kinase-MM	MAK33 IgG1	Cardiac disease, mitochondrial disorders, *inflammatory myopathies*, myasthenia, polymyositis, McArdle's disease	*A thaliana*		A thaliana 2S2 seed storage protein SP	+ 0 02–0 4% of TSP of fresh leaf extract (10–12% of TSP of intercellular fluid)	De Wilde *et al* (1996)
	Fab fragment	NMJ disorders, muscular dystrophy, ALS, hypo- and hyperthyroid disorders	*A thaliana* *N tabacum*	35 S promoter		+ 1 3% of TSP 0 044% of TSP	De Neve *et al* (1993)
	MAK33 scFv	central core disease, acid *maltase deficiency*, myoglobinuria, rhabdomyolysis, motor neurone diseases, rheumatic diseases, and others that create elevated or reduced levels of creatine kinases	*N tabacum* SR1	35S promoter	No SP A thaliana 2S2 seed storage protein SP	+ 6–25 ng scFv/mg TSP + 8–55 ng scFv/mg TSP	Bruyns *et al* (1996)
	MAK33 Fab fragment		*A thaliana*	35 S promoter	A thaliana 2S2 seed storage protein SP SP + KDEL	+ 6 53% of TSP (0 02% of TSP in seed) + 5 9% of TSP (0 015% of TSP in seed)	Peeters *et al* (2001)
Tumour's surface Ig	38C13 scFv	B-cell lymphoma treatment	*N benthamiana*	TMV coat protein promoter	Rice α-amylase SP	+ 60 µg/mL of intercellular medium	McCormick *et al* (1999)
Hepatitis B virus surface antigen (HBsAg)	scFv	Immunoaffinity purification of *recombinant* HBsAg	*N tabacum* cv petit Havana SR1	35 S promoter	No SP Sweet potato Sporamin SP Sporamin SP + PP Sporamin SP + KDEL	– + 0 031% of TSP + 0 032% of TSP + 0 22% of TSP	Ramirez *et al* (2002)
Zearalenone (mycotoxin)	scfv	Passive immunization of animals in their feed	*A thaliana* (ecotype Columbia)	Inducible *Lac* promoter	No targeting signal Plant PR1-b SP	– +	Yuan *et al* (2000)

ALS, amyotrophic lateral sclerosis, FLW, fresh leaf weight, KDEL, endoplasmic reticulum retention signal, MSP, murine signal peptide, NMJ, neuronuscular junction, PP, propeptide, SP, signal peptide, TSP, total soluble protein

Table 2 Therapeutic antibodies clinically validated

Drug	Company	Indication	Molecule	Antigen	Year approved
ReoPro	Eli Lilly	Acute coronary syndromes	Abciximab Chimeric human murine Fab fragment	Integrine GPIIb/IIIa	1994
Orthoclone	Janssen	Acute kidney transplant rejection	Muromonab Murine monoclonal antibody	CD3	1986
Rituxan MabThera	Genentech	Non-Hodgkin's lymphoma	Rituximab Chimeric human/mouse monoclonal antibody	CD20	1997
Herceptin		HER2+ metastatic breast cancer	Trastuzumab Humanized antibody	P185 (HER2/Neu)	1998
Simulect	Novartis	Kidney transplant rejection	Basiliximab Chimeric human/mouse monoclonal IgG	Interleukin 2 receptor α-chain (CD25)	1997
Remicade	Centocor	Crohn's and rheumatoid arthritis	Infliximab Chimeric human/mouse monoclonal antibody	TNFα	1998/1999
Zenepax	Roche, PDL	Kidney transplant rejection	Daclizumab Humanized antibody	CD25 (IL-2R)	1997
Synagis	Medimmune	Prevention of RSV infection	Palivizumab Humanized antibody	F protein of syncytial respiratory virus	1998
Mylotarg/AHP	Celltech	Acute myeloid leukaemia	Gemtuzumab Humanized antibody that is likened to the toxin calicheamicin	CD33	2000
Campath	ILEX	Chronic lymphocytic leukaemia	Alemtuzumab Humanized antibody	CD52	2001

RSV, respiratory syncytial virus

production of 1 kg of antibody per year) (Steiner, 2000). Currently, the capacity available for the production of monoclonal antibodies does not exceed 1000 kg per year. This means that almost all the current production capacity is used to provide sufficient amounts of the first 10 monoclonal antibodies clinically validated today (Table 2). Therefore, the pharmaceutical industry will need to invest a considerable amount of money to allow the commercialization of future monoclonal antibodies produced in culture units of CHO cells, which are currently undergoing clinical tests. Even in these conditions, and considering the delays needed for the construction of such equipment, it can be estimated that, in 2010, the production capacity would be, at best, 10 000 kg of antibodies with an estimated demand of 50 000 kg (Gura, 2002).

In this context, pharmaceutical companies are deeply interested in transgenic production systems which enable high production levels of therapeutic antibodies. Apart from cultured mammalian cells, only transgenic animals and transgenic plants are able to assemble, via disulphide bridges, the light and heavy chain constituents of an antibody (Figure 1). In particular, plant cells can reproduce the complexity of these proteins, as illustrated in 1989 by the first production of functional antibodies in plants (Hiatt *et al.*, 1989). Since this pioneering demonstration from Dr Hiatt's group, many antibodies, or fragments of antibodies, have been produced for therapeutic purposes in various plant systems, in particular antibodies directed against human immunoglobulins (Igs) (Khoudi *et al.*, 1999), against an antigen from *Streptococcus mutans* (Ma *et al.*, 1995; Smith and Glick, 2000), against a human creatine kinase (Conrad *et al.*, 1998) or against a tumoral antigen expressed in colon cancer (Vaquero *et al.*, 1999). Antibody fragments or diabodies have also been produced in plants, in particular against tumour-specific antigens (Stöger *et al.*, 2000; Vaquero *et al.*, 2002) (Figure 1).

Therefore, the use of transgenic plants could be a solution to the need for a scale-up in the production capacity of therapeutic antibodies. Indeed, even with relatively low expression levels of total soluble proteins (Hiatt *et al.*, 1989; Ma *et al.*, 1995) (Table 3), the production capacity of recombinant antibodies in transgenic plants is almost unlimited, as it depends only on the surface dedicated to the plant culture. Although dependent on the stability of the protein of interest and on the plant used to produce it (tobacco, corn, soybean or alfalfa), it is estimated that a plant 'bioreactor' will allow

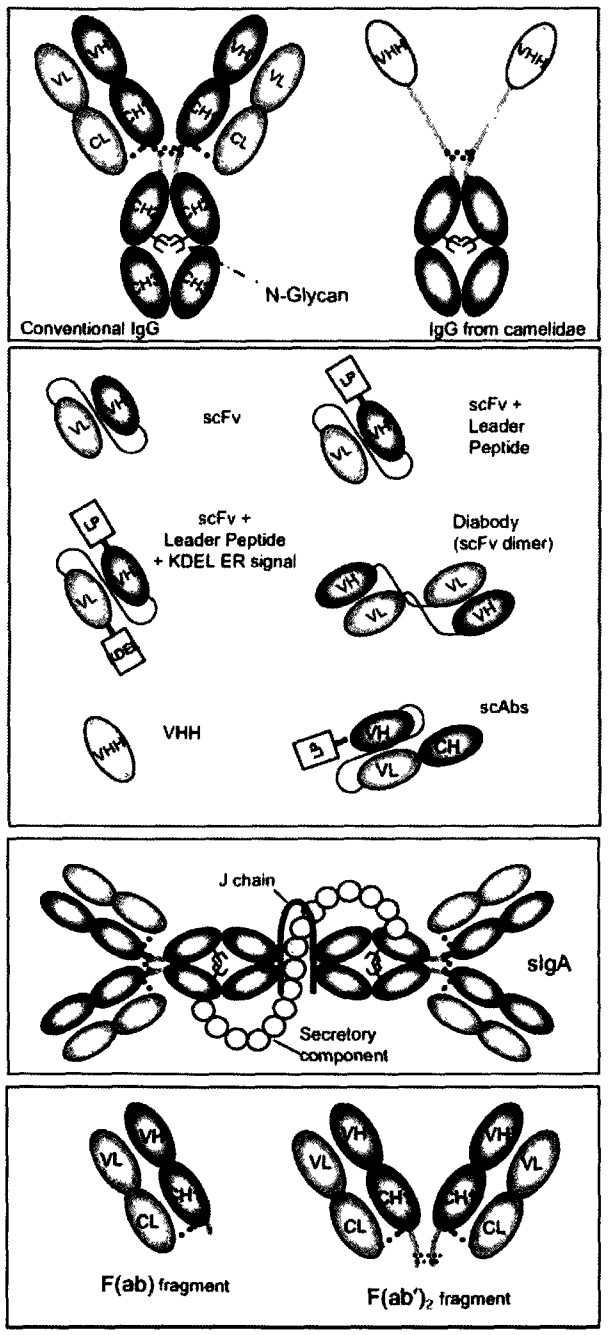


Figure 1 Various antibodies and antibody fragments produced in plant systems. In a conventional immunoglobulin G (IgG), the constant heavy-chain (CH1, CH2, CH3) and the variable heavy-chain (VH) domains are shown in orange, and the constant light-chain (CL) and variable light-chain (VL) domains in green. In the recombinant single-chain Fv fragment (scFv), the variable domains are joined by a peptide linker scFvs can be complexed into dimers (diabodies), trimers or tetramers (not illustrated) Diabodies have two functional antigen binding domains with similar or distinct antigen specificities Some camelidae IgGs lack a light chain Their antigen-binding domains consist only of the heavy-chain variable domain (VHH). The VHH antibody fragment is highly soluble and stable A secretory IgA (sIgA) is a dimeric IgA composed of 10 different polypeptides. four light chains, four heavy chains, a J chain that facilitates dimerization and a secretory component The F(ab) fragment contains the light chain and the VH and CH1 domains of the heavy chain $F(ab')_2$ is divalent and contains two F(ab) fragments linked by a disulphide bond

Table 3 Comparison of currently available recombinant protein production systems

	Bacteria	Yeasts	Mammalian cells	Insect cells	Transgenic mammals	Transgenic plants
Risk of pathogenicity	Yes	Unknown	Yes	Yes	Yes	Unknown
Production cost	Medium	Medium	High	High	High	Low–medium 10–50-fold lower than the same protein produced by *E. coli*
Time effort	Low	Medium	High	High	High	High
Scale-up cost	High	High	High	High	High	Low (unlimited biomass)
Production	Limited	Limited	Limited	Limited	Limited	Worldwide
Production yield (i.e. antibodies)	Medium 0.1–2 g/L	High > 0.25 g/L (scFv)	Medium to high 0.4–2 g/L	Medium to high 5–70 mg/L	High 4–14 g/L	High 500–1000 g/acre/cycle
Folding accuracy	Low	Medium	High	High	High	High
Glycosylation status						
N-glycosylation	No	Yes (unusual)	Yes	Yes	Yes	Yes
Site of glycosylation		Not conserved	Conserved	Conserved	Conserved	Conserved
Non-human sugar residues		Yes	yes	yes	Yes	Yes
Product homogeneity	Low	Medium	Medium	Low	Low	Medium to high
Protein complexity (i.e. antibody)	Fab	Fab	IgG, IgM	IgG	IgG	IgG, sIgA
Delivery vehicle	No	No	No	No	Yes	Possible
Storage	–20 °C	–20 °C	N_2	N_2	N_2	Room temperature
Distribution	Feasible	Feasible	Difficult	Difficult	Difficult	Easy
Ethical concerns	Medium	Medium	Medium	Medium	High	Medium
Companies	GenWay Biotech Inc Genentech ATG Laboratories Paragon Bioservices Biological Mimetics	Paragon Bioservices ApoLife, Inc	GenWay Biotech Inc Paragon Bioservices Cell Trends Acceptys, Inc Lonza Biologics Micromet/Enzon Biological Mimetics Genentech	ATG Laboratories Paragon Bioservices Cell Trends Biological Mimetics	Nexia Biotechnologies GTC Biotherapeutics PPL Therapeutics Abgenix Pharming	Biolex Incorporated ProdiGene Greenovation Biotech GmbH SemBioSys Medicago Inc Meristem Therapeutics Phytomedics, Inc Epicyte Pharmaceuticals

the production of recombinant proteins in the range of kilograms per hectare, regardless of the plant material considered (Austin *et al.*, 1994; Khoudi *et al* , 1999). Another advantage of antibody production in transgenic plants, compared with classical production in culture units of mammalian cells, is that it would cost 500 times less (Table 3). Considering the expense and difficulty of producing antibodies by conventional means, it seems that production in crop plants is one of the most promising options for large-scale antibody production.

Only transgenic mammals could present an antibody production capacity equivalent to that estimated for transgenic plants. The efficiency of this production system has been well documented, as about 100 recombinant proteins have already been expressed in the milk of transgenic animals (Houdebine, 2002). However, this system also presents some limitations as the production of some recombinant proteins, such as therapeutic antibodies, might disturb the metabolism of the transgenic animal. Moreover, although PMPs cannot be contaminated by human pathogens, doubts still exist about the contamination of recombinant proteins, expressed in the milk of transgenic mammals, by human-transmissible pathogens.

Many plant systems are available for the production of recombinant antibodies

Several plant species have been successfully used to produce biologically active antibodies. In tobacco, alfalfa and some other species, expression is performed in leaves, whereas, in potato, corn, rapeseed, safflower, soybean, wheat or rice, the production and accumulation of recombinant antibodies occur in the tubers or in the seeds Both production strategies present their advantages and drawbacks. Leaves present an active and complex metabolism which offers many possibilities, but they also contain a substantial protease activity which limits the accumulation of some proteins. Seeds present the advantage of offering a lower water content, and therefore represent a more stable accumulation site. In addition to the organ-specific storage of PMP(s), the increasing amount of information on protein targeting in a plant cell also allows for the storage of a protein of interest in various subcellular compartments. Up to now, most recombinant antibodies produced in plants have been secreted in the apoplast based on the efficiency of a signal peptide to target a foreign protein for secretion (Table 3). However, recent results have shown that the H/KDEL-mediated retention in the endoplasmic reticulum (ER) could present several advantages, including a strong increase in the stability of the recombinant protein (Wandelt *et al.*, 1992; Pueyo *et al.*, 1995; Tabe *et al.*, 1995; Conrad and Fiedler, 1998; Pagny *et al.*, 2003b) and prevention of the addition of complex (i.e. immunogenic) N-glycans on plant-made antibodies (Finnern *et al.*, in press) Targeted expression of PMP(s) in the chloroplast also offers many advantages. For instance, by combining high expression levels and low proteolytic activity, a protein of interest expressed in this organelle could represent up to 20% of total chloroplast proteins (Maliga, 2003). This organelle looks particularly well suited to the production of very simple antigen binding molecules, such as VHH from camelidae IgG (Figure 1) (Jobling *et al.*, 2003), but, surprisingly, tobacco chloroplasts are also capable to properly fold complex proteins with disulphide bridges, such as human somatotropin (Staub *et al.*, 2000) and full-size antibodies (Daniell *et al.*, 2001).

A better knowledge of the signals and mechanisms responsible for protein targeting to the protein storage vacuole will help further investigations on the advantages and limitations of PMP(s) storage in this compartment (Paris and Neuhaus, 2002).

Plant-specific purification strategies

In all heterologous production systems, the recombinant protein will have to be extracted and purified from endogenous proteins of the producing organism. Protein purification is known to be a key stage in the preparation of a biopharmaceutical and it could represent more than 80% of its production cost. Antibody purification from plant protein extracts is generally achieved using existing technologies, such as affinity chromatography methods based on specific interactions with antigens, *Staphylococcus aureus* Protein A or *Streptococcus* sp. Protein G. The targeted expression of PMP(s) into specific subcellular compartments also offers plant-specific strategies to simplify the first steps of purification. For example, in oleaginous plants, the recombinant protein can be fused to a protein (oleosin) anchored in the membrane of lipid bodies, organelles that accumulate in the seed during maturation (Figure 2A). By combining this fusion with an expression targeted to the seed, researchers have established a simple expression/purification system in which the recombinant protein is recovered with the lipids after a centrifugation step. The recombinant protein, cleaved by targeted proteolysis, is then extracted from the lipid fraction by a second phase partitioning. This strategy has been used in rapeseed for the production of hirudin, an anticoagulant from leach salivary glands (Parmenter *et al.*, 1995; Boothe *et al* , 1997). Recently, it has been adapted to the production of recombinant antibodies in seeds of another oilseed plant, safflower (Seon *et al.*, 2002) As shown in Figure 2(B), the transgenic plant synthesizes and accumulates two recombinant

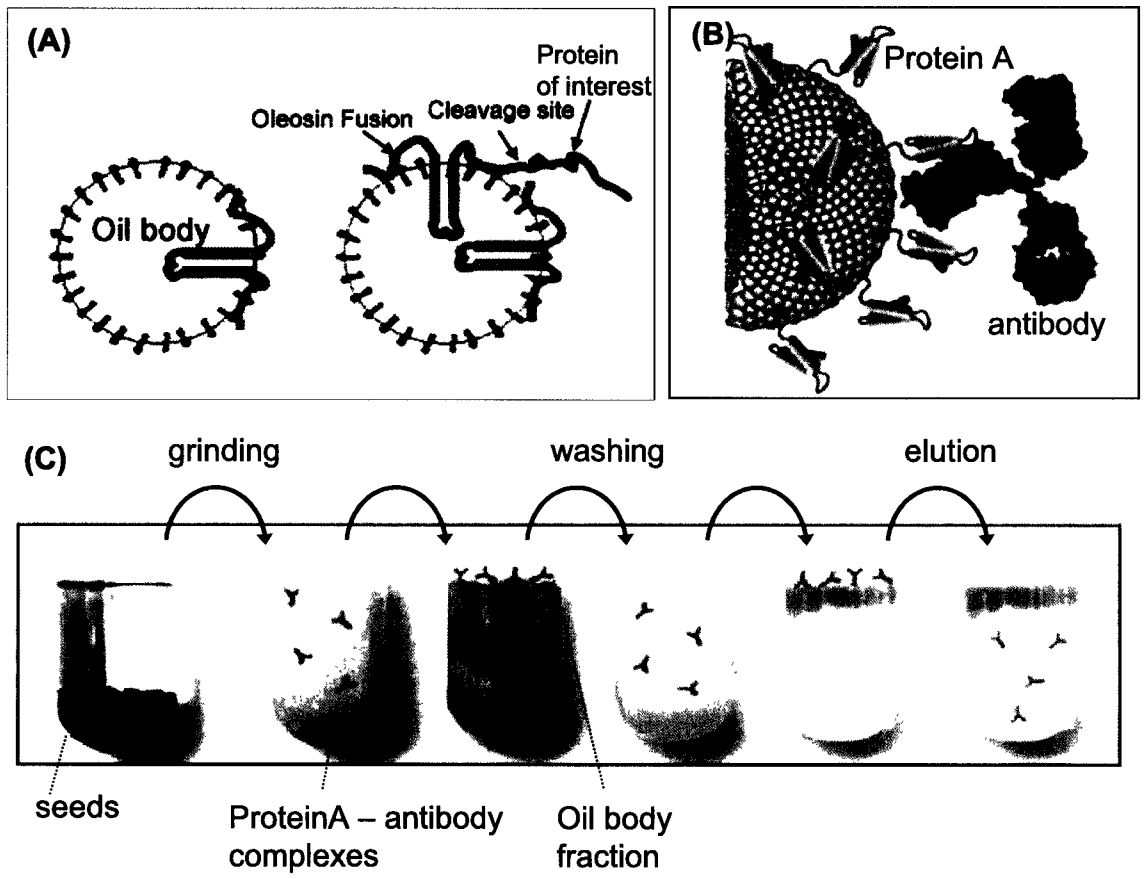


Figure 2 Strategy for the purification of a recombinant antibody by targeting to lipid bodies Panel A Anchoring of the oleosin molecule in the membrane of a native oil body (left) Recombinant lipid body presenting in its membrane an oleosin molecule fused to a protein of interest (right) Panel B After grinding the seeds, the recombinant antibodies form complexes with the protein A molecules fused to oleosin and expressed on the surface of the recombinant lipid bodies Panel C Purification by centrifugation of antibodies from safflower seeds where the two recombinant proteins are stored· antibody and protein A fused to oleosin (Adapted from Seon *et al.*, 2002 and http //www sembiosys ca/stratosome.html)

proteins in two distinct cellular compartments of the seed: an antibody and a protein presenting a high affinity for this antibody, protein A fused to oleosin. The protein A-fused oleosin and the antibody form complexes when the seed is ground. These complexes are easily isolated, and purified with the lipid phase from the extract. Eventually, the antibodies are released after dissociation of the complexes by an acidic treatment (Figure 2C). Similar approaches, yielding simplified purification and a reduced proteolysis of the recombinant protein, have been developed using the advantages of protein expression in transgenic chloroplasts. As previously described for Bt toxin (De Cosa *et al.*, 2001), due to its high expression level, human serum albumin (HSA) aggregates and forms inclusion bodies in tobacco chloroplasts. Inclusion bodies are easily purified from soluble cellular proteins, and HSA recovered from inclusion bodies is solubilized and *in vitro* refolded (Daniell and Dhingra, 2002).

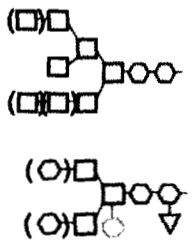
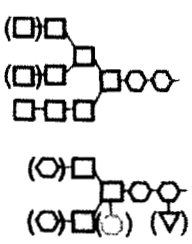
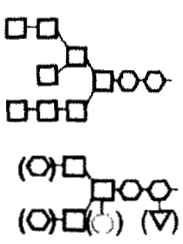
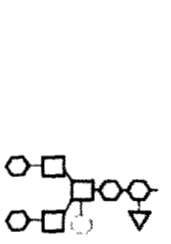
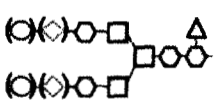
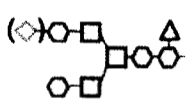
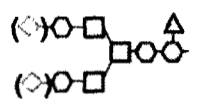
Conformity of recombinant antibodies produced in plant systems

Plant cells are able to reproduce the complexity of human proteins. The homologies shared by the protein biosynthesis and maturation machinery in animal and plant cells are well illustrated through the ability of plants to produce various types of recombinant antibodies, such as IgGs or secretory IgAs (sIgAs) (Table 1) Antibodies are complex molecules. Immunoglobulins of the IgG class are tetramers consisting of two identical polypeptides of 450 amino acids (heavy chains) and two identical polypeptides of 250 amino acids (light chains). These four IgG-constituent polypeptides are linked together by several disulphide bridges. The complexity of an sIgA is even greater, as these immunoglobulins are composed of two IgA molecules linked together by two polypeptides (Figure 1). The assembly of an sIgA requires the action of

two different cell types in mammals. sIgA, IgGs and IgAs have been produced with success, in a biologically active form, in transgenic plants. This illustrates the ability presented by the plant cell machinery to assemble mammalian proteins, even when the latter are extremely complex However, further work is still needed to explain why secretory IgA/IgGs, expected to be secreted in the tobacco apoplast, have been found in different subcellular compartments, such as the vacuole and the ER (Frigerio *et al.*, 2000). This heterogeneous distribution of a recombinant antibody in a plant cell could result from either improper folding or targeting. Unfortunately, it could induce a large structural heterogeneity in the PMP due to different proteolytic maturations occurring on the proteins in the ER, vacuole and apoplast. The N-glycan maturation is also reflective of the localization of a glycoprotein in the secretory pathway (Lerouge *et al.*, 1998), and the strong heterogeneity observed in the glycosylation of IgGs produced in tobacco could also be explained through this heterogeneous distribution (Cabanes-Macheteau *et al.*, 1999; Bakker *et al.*, 2001). In contrast, an IgG produced in alfalfa presents a very homogeneous N-glycan structure, which could be explained by a higher efficiency of the secretion and/or folding machinery in this plant expression system (Bardor *et al.*, 2003b).

Antibodies produced in transgenic plants differ from mammalian antibodies: immunogenic glyco-epitopes

The correct folding and assembly of plant-made antibody molecules within the ER, through interactions with a number of chaperones, processing and glycosylation enzymes, illustrate that co- and post-translational protein maturation events are similar in plants and mammals. However, as illustrated in Figure 3, antibodies produced in mammalian cells differ in their N-glycosylation from those produced in a plant system. This is illustrated with the monoclonal antibody Guy's 13, which is specific for an adhesin from *Streptococcus mutans*, the major pathogen contributing to the development of dental caries. When produced in mammalian cells, this IgG1 is glycosylated on its two N-glycosylation sites with oligosaccharidic structures (N-glycans) presenting an α(1,6)-fucose residue and about 10% terminal sialic acid. When produced in transgenic tobacco cells, the Guy's 13 antibody is also glycosylated on both N-glycosylation sites. However, the N-glycans borne by this antibody are of the high-mannose type (common structures to plants and mammals) and complex type, with typical characteristics for plants, such as β(1,2)-linked xylose and α(1,3)-linked fucose (Cabanes-Macheteau

	Nicotiana tabacum *Guy's 13*	*Nicotiana tabacum* cv Samsum *Mgr48*	*Nicotiana tabacum* Cv petit Havana SR1 *cPIPP*	*Medicago sativa* *C5-1*
Plants				
Mammals			ND	
	Cabanes-Macheteau *et al.*, 1999	Bakker *et al.*, 2001	Finnern *et al.*, in press	Bardor *et al.*, 2003b

○ GlcNAc □ Man ◌ β1,2Xyl ▽ α1,3Fuc ◇ β1,4 Gal ○ NeuAc α1,6Fuc

Figure 3 Structure of glycans N-linked to immunoglobulin G (IgG) molecules expressed in hybridoma and transgenic plants

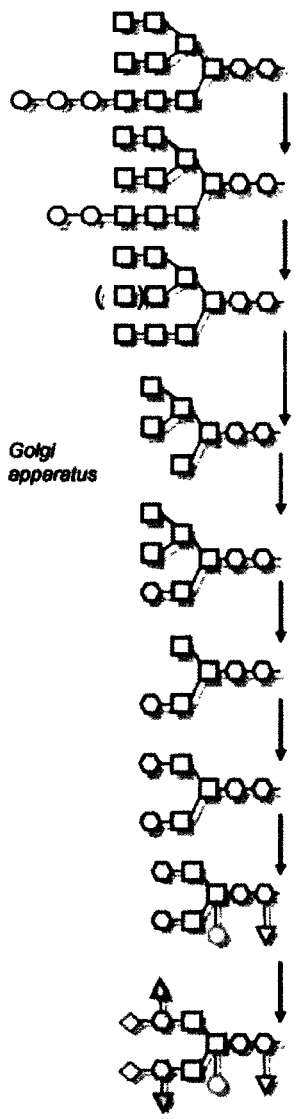
et al., 1999). Similar glycan heterogeneity and structural characteristics have been described for another monoclonal antibody (Mgr48) produced in tobacco (Bakker *et al.*, 2001). In contrast with the IgG expressed in tobacco plants, the N-glycosylation of an alfalfa-derived antibody (C5-1) is restricted to a predominant mature oligosaccharide chain having a core α(1,3)-fucose, a bisecting β(1,2)-xylose and two terminal GlcNAc residues. Although the homogeneity of glycosylation could differ from one plant expression system to the other, all plant species used so far to produce PMPs have the capacity to associate β(1,2)-xylose and α(1,3)-fucose residues on to complex N-glycans (Cabanes-Macheteau *et al.*, 1999; Bakker *et al* , 2001; Bardor *et al.*, 2003b; Samyn-Petit *et al.*, 2003). These residues are constituents of glyco-epitopes, known to be important IgE binding carbohydrate determinants of plant allergens (Aalberse *et al.*, 1981; Faye and Chrispeels, 1988; Van Ree and Aalberse, 1995; Garcia-Casado *et al.*, 1996; Van Ree *et al.*, 2000). More importantly, it has recently been found that a plant N-glycan containing these glyco-epitopes not only shows IgE binding, but also causes mediator release by human basophils, when at least two of these N-glycans are present on the same protein (Westphal *et al.*, 2003). We and others have also reported that the immunization of goats (Kurosaka *et al.*, 1991) or rabbits (Faye *et al.*, 1993) with plant glycoproteins elicits the production of antibodies specific for these glyco-epitopes containing β(1,2)-xylose or α(1,3)-fucose. More recently, *in vivo* experiments using BALB/c mice have shown that plant-made antibodies produced in tobacco do not elicit immunological response against their *N*-linked glycans of plant origin (Chargelegue *et al.*, 2000). Altogether, these data obtained in laboratory mammals raise the question of the immunogeneity of these glyco-epitopes in the context of a human therapy based upon PMPs. We recently addressed this question by reinvestigating their immunogenicity in rodents. We found that, in C57BL/6 mice and rats, but not using BALB/c mice, immunization with a model glycoprotein, horseradish peroxidase, elicited the production of antibodies specific for α(1,3)-fucose- and β(1,2)-xylose-containing glyco-epitopes. Furthermore, we demonstrated that about 50% of non-allergic human blood donors contain in their sera antibodies specific for the β(1,2)-xylose glyco-epitopes, whereas 25% have antibodies against the α(1,3)-fucose glyco-epitope (Bardor *et al.*, 2003a). These antibodies probably result from sensitization to environmental antigens. Although the immunological significance of anti-α(1,3)-fucose and anti-β(1,2)-xylose antibodies is too speculative at the moment, the presence of such antibodies may, at least, induce a rapid immune clearance of glycosylated PMPs from the circulation, which may greatly compromise their effectiveness as *in vivo* therapeutic agents. In addition to this accelerated clearance, the clinical effects resulting from the immune response caused by the administration of plant-derived glycoproteins in allergic or non-allergic patients are also questionable. As a consequence, for safety concerns related to the use of plant-derived therapeutic proteins, further experiments should be carried out in an appropriate animal model, as well as in human volunteers, by administering a therapeutic glycoprotein produced in a plant and analysing the immune responses to the plant glyco-epitopes in allergic and non-allergic populations.

Interestingly, even when antibodies are produced in mammalian expression systems, they also contain non-human sugar residues, such as the N-glycosylneuraminic acid (Neu5Gc) form of sialic acid (antibodies produced in CHO cells and in milk) or terminal α(1,3)-galactose (antibodies produced in murine cells). It has been shown that antibodies containing these sugar residues can also provoke undesirable side-effects, including an immune response in humans. Several examples of the genetic manipulation of CHO cells have been attempted to reduce the Neu5Gc level in recombinant glycoproteins (see Chenu *et al.*, 2003 for a recent paper). Similarly, the following part of this paper describes the current efforts carried out to prevent the association of immunogenic N-glycans to PMPs.

Towards the humanization of antibody glycosylation in plants

In plants, as in other eukaryotic cells, N-glycosylation starts in the ER, in a cotranslational way, by the addition of an oligosaccharide precursor ($Glc_3Man_9GlcNAc_2$) to specific asparagine residue constituents of the potential N-glycosylation-specific sequences, Asn-X-Ser/Thr, borne by the elongating polypeptide. Once transferred on to the nascent protein, and while the secreted glycoprotein is transported along the secretory pathway, the oligosaccharide undergoes several maturation reactions involving the removal or addition of residues in the ER and the Golgi apparatus (Figure 4). It is only in the late Golgi apparatus that plant and mammalian N-glycan maturation differs, which results in the absence of α(1,6)-linked fucose and sialic acids and the presence of bisecting β(1,2)-xylose and core α(1,3)-fucose in the N-glycans of PMPs, as illustrated in Figure 5.

To be able to fully use the potential plants can offer for the production of recombinant antibodies, it becomes necessary to inhibit these plant-specific maturations in order to obtain 'humanized' non-immunogenic N-glycans. Numerous projects are currently in progress in order to prevent the addition of



	Enzymes	Organisms	References
A	Mannosyl-oligosaccharide glucosidase I (EC 3.2.1.106)	*A. thaliana*	Boisson *et al* . 2001
B	Mannosyl-oligosaccharide glucosidase II	*S. tuberosum*	Taylor *et al*., 2000
C	ER-mannosyl-oligosaccharide 1, 2 alpha mannosidase (EC 3.2.1 -)		
D	Mannosyl-oligosaccharide 1.2 alpha mannosidase (EC3 2.1.113)	*Glycine max.*	Nebenfuhr *et al*., 1999
E	α 1,3 mannosyl-glycoprotein beta1.2 N-acetylglucosaminyltransferase (EC 2.4.1.101)	*A. thaliana* *N. tabacum* *S. tuberosum*	Wenderoth *et al*., 2000 Strasser *et al*., 1999
F	Mannosyl-oligosaccharide 1,3-1,6 alpha mannosidase (EC 3.2.1.114)		
G	α 1,6 mannosyl glycoprotein beta 1.2 N acetylglucosaminyl transferase (EC 2.4.1.143)	*A. thaliana*	Strasser *et al*., 1999
H	β1,2 xylosyltransferase (EC2.4.2.38)	*A. thaliana* *M. sativa* *P. Patens*	Strasser *et al*, 2000 Pagny *et al*., 2003 Sourrouille *et al*. In preparation Koprinovova *et al*., 2003
I	α 1,3 fucosyltransferase (EC 2.4 1.214)	*A. thaliana* *V. radiata* *M. sativa* *P. patens*	Leiter *et al*., 1999 Wilson *et al*., 2001 Bakker *et al*., 2001 Sourrouille *et al*., in preparation Koprinovova *et al*., 2003
J	β 1,3 galactosyltransférase (EC2.4.1.-)		
K	α 1,4 fucosyltransferase (EC2.4.1.-)	*A. thaliana* *M. sativa* *L. esculentum* *B. vulgaris*	Leonard *et al*., 2002 Wilson *et al*., 2001 Sourouille *et al*., in prepapration Bakker *et al*., 2001

Figure 4 Processing of N-glycans in plants N-glycosylation of plant proteins starts in the endoplasmic reticulum (ER) with the transfer of an oligosaccharide precursor $Glc_3Man_9GlcNAc_2$ on specific Asn residues of the nascent polypeptide chain This precursor is then modified by glycosidases and glycosyltransferases mainly in the ER and the Golgi apparatus during the transport of the glycoprotein downstream of the secretory pathway. Glycosidases and glycosyltransferases responsible for plant N-glycan maturation are indicated from A to K in the left panel. Most of these plant N-glycan processing enzymes have recently been cloned from different plants as indicated in the right panel.

immunogenic glycans on to PMPs. One of the most drastic approaches is to prevent the addition of immunogenic N-glycans on plant-made antibodies by inactivating N-glycosylation sites through the mutation of Asn or Ser/Thr. In most cases, this strategy will not inactivate the antigen binding function of antibodies. However, many pharmaceuticals, including antibodies used for their effector functions, such as the triggering of the immune response (Wright and Morrison, 1994), require glycosylation for an increased *in vivo* activity and stability. A recent observation has also shown that the addition of N-glycans to several recombinant protein or glycoprotein therapeutics increases their biological activity and half-life. This further illustrates a current tendency in glyco-engineering to increase, and not to reduce, the number of glycosylation sites on recombinant pharmaceuticals (Elliott *et al*., 2003).

Promising results have been obtained in the production of plant-made glycosylated therapeutic proteins bearing

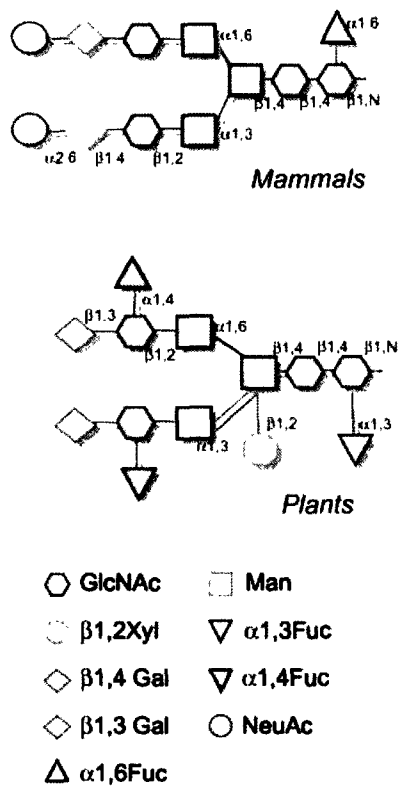


Figure 5 Plant and mammalian N-glycans have different structures. The first steps of N-glycan maturation are common to plants and mammals Only the late steps differ, when glycosyltransferases specific for plants or for mammals act on the N-glycan. As illustrated here for typical plant and mammalian biantennary complex N-glycans, these differences in the processing machinery result in the absence of sialic acids in the terminal position of the antennae and the presence of a bisecting β(1,2)-xylose and an α(1,3)-fucose residue instead of α(1,6)-fucose linked to the proximal N-acetylglucosamine of the N-glycans of plant-made pharmaceuticals (PMPs).

non-immunogenic N-glycans by storing antibodies within the ER, i.e. upstream of the compartment where plant-specific maturations take place. Other strategies, involving the inhibition of Golgi glycosyltransferases and the expression of mammalian glycosyltransferases within this compartment, are now under investigation. What has currently been achieved is detailed below.

Retention in the ER: control of glyco-epitope formation vs. PMP clearance

The structural analysis of plant ER-resident proteins has shown that natural reticuloplasmins bear high-mannose-type N-glycans (Navazio *et al.*, 1996, 1998; Pagny *et al.*, 2000). These oligosaccharide structures are common to plants and mammals, and therefore are probably not immunogenic. This observation has suggested a strategy to prevent the association of immunogenic glycans to PMPs. This strategy consists in the storage of recombinant proteins within the ER, i.e. upstream of Golgi cisternae, where immunogenic glyco-epitopes are added to maturing plant N-glycans. It was first shown that the addition of H/KDEL sequences at the C-terminal end of a recombinant protein is sufficient for its retention in the plant ER (Gomord *et al.*, 1997, 1999 for a review). By this approach, we have shown that a model secretory protein (i.e. cell wall invertase) fused to an HDEL retrieval sequence was efficiently retained within the ER. A detailed structural analysis has shown that this cell wall invertase bearing the HDEL sequence (invertase-HDEL) displays mainly high-mannose-type glycans as well as a detectable amount of glycans containing the β(1,2)-xylose epitope (Pagny *et al.*, 2000). This indicates that a small amount of this protein has first been transported across the secretory pathway down to the medial Golgi, where β(1,2)-xylosyltransferase is located (Pagny *et al.*, 2003a), from which it is then retrieved and transported back to the ER.

Similar results have recently been obtained with a human monoclonal antibody containing KDEL sequences fused at the C-terminal end of both heavy chains, and expressed in tobacco plants (Ko *et al.*, 2003). As observed for invertase-HDEL, most glycans N-linked to this antibody were of the high-mannose type with six to nine mannose residues, but a fraction (about 10%) of these glycans contained the immunogenic β(1,2)-xylose glyco-epitope (Figure 6). However, this antibody was not α(1,3)-fucosylated, a glycan modification occurring in the *trans*-Golgi side (Fitchette-Lainé *et al.*, 1994).

The retrieval efficiency is further increased when the ER retention sequence is fused to both heavy and light chains of the antibody. This was clearly illustrated with a chimeric mouse-human antibody expressed in tobacco and harbouring four KDEL retrieval signals on the fully assembled H2L2 form. As shown in Figure 6, this antibody presents exclusively high-mannose-type N-glycans with six to nine mannose residues (Finnern *et al.*, in press), indicating a very efficient recycling based on glycan maturation limited to enzymes located in the ER and *cis*-Golgi, such as α-mannosidase I (Nebenfuhr *et al.*, 1999).

Therefore, preventing the association of immunogenic N-glycans to PMPs through the fusion to ER retention signals is possible. However, when compared with their mammalian homologues, antibodies produced using this strategy present a very low stability after their injection in mice (Ko *et al.*, 2003). These antibodies with high-mannose N-glycans are probably rapidly degraded after binding to the mannose receptor and endocytosis by macrophages, as previously observed for

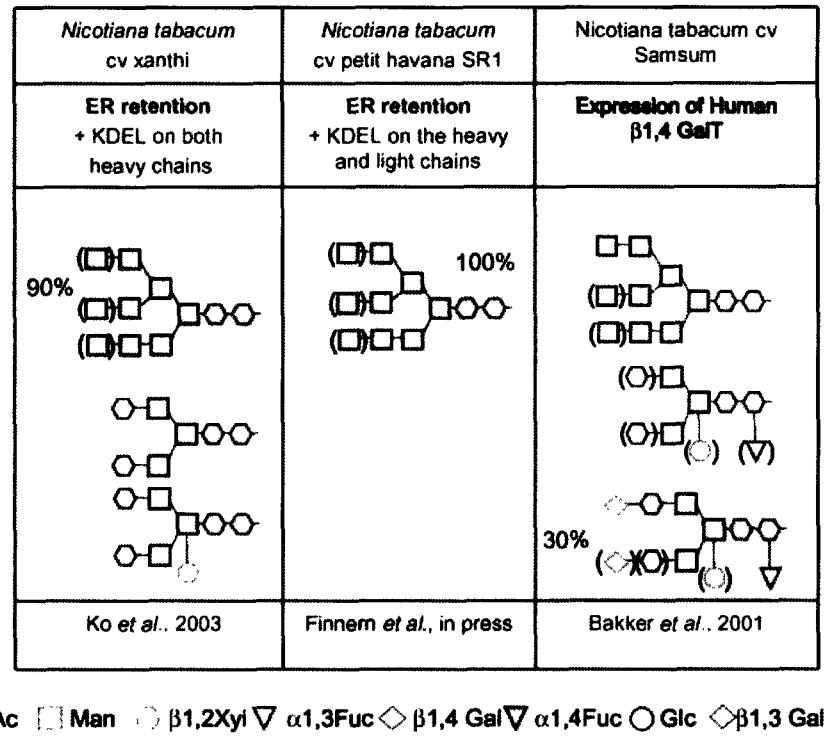


Figure 6 Some successful strategies towards plant N-glycan humanization This figure illustrates glycosylation of plant-made antibodies produced with KDEL retrieval signals or in tobacco plants expressing a human β(1,4)-galactosyltransferase An antibody fused with two (left panel) or four (middle panel) KDEL-ER retention signals is mostly or exclusively glycosylated with high mannose and probably non-immunogenic N-glycans When an antibody is produced in tobacco plants expressing the human galactosyltransferase (right panel), 30% of its N-glycans show terminal N-acetyllactosamine sequences identical to those borne by this antibody when produced in hybridoma cells

antibodies produced in Lec 1 mutant CHO cells (Wright and Morrison, 1998). In a similar clearance assay following intramuscular injection, a plant-made antibody with complex N-glycans (Bardor *et al.*, 2003b) has been shown to be as stable as its mammalian homologue in the bloodstream of mice (Khoudi *et al.*, 1999). This indicates that, in contrast with high-mannose N-glycans, matured plant N-glycans are not recognized by the mannose receptor. However, BALB/c mice used to study *in vivo* the stability of plant-made antibodies definitely provide the most inappropriate animal model to appraise both plant N-glycan immunogenicity and glycosylated PMPs' clearance in blood via carbohydrate-specific antibodies.

En route to the inactivation of plant glycosyltransferases

The analysis of an *Arabidopsis* mutant has shown that the inactivation of only one glycosyltransferase, N-acetylglucosminyltransferase I (GnTI), is sufficient to block the biosynthesis of complex N-glycans (von Schaewen *et al.*, 1993). This glycosyltransferase has been cloned from several plants (Figure 4). However, the expression of antisense GnTI in tobacco and potato plants has only shown a decrease in complex N-glycan biosynthesis in these plants (Wenderoth and von Schaewen, 2000).

Independent of its low efficiency in the prevention of immunogenic N-glycan formation, this pioneer study has stimulated the interest of several laboratories and molecular farming companies for plant glycosyltransferases. Many of these enzymes, in particular 'targets' for inactivation, such as β(1,2)-xylosyltransferase and α(1,3)-fucosyltransferase, have been cloned within the past 5 years in several plant expression systems (Figure 4). In the near future, the development of strategies allowing an efficient inhibition of these glycosyltransferases will prevent glyco-epitope formation on therapeutic proteins produced in higher plants. Recent results obtained in the moss *Physcomitrella patens* have paved the way to inactivation strategies in higher plants N-linked glycosylation of mosses is highly similar to that in higher plants (Viëtor *et al.*, 2003), and its genes for α(1,3)-fucosyltransferases and β(1,2)-xylosyltransferase show 50% and 38% identity, respectively, with those already cloned from *A. thaliana*. *P. patens* is the only known plant system which shows a

high frequency of homologous recombination. This strategy has been used to knock out α(1,3)-fucosyltransferases and β(1,2)-xylosyltransferase genes, permitting the disappearance of plant-specific glyco-epitopes without any effect on protein secretion in this moss (Koprinovova *et al.*, 2003).

As described for their mammalian counterparts, plant glycosyltransferases are type II membrane proteins. This prediction has been confirmed through the detailed characterization of β(1,2)-xylosyltransferase from *A. thaliana*. The first 36 amino acids of this glycosyltransferase (i.e. cytosolic tail + transmembrane domain) are sufficient for its Golgi retention, but also contain information on sub-Golgi compartment targeting (Pagny *et al.*, 2003a). The analysis of several other glycosyltransferases is currently providing a panel of specific signals that are sufficient for protein targeting within the different Golgi subcompartments. These signals will help to target exogenous glycosyltransferases in the plant Golgi apparatus for an optimal efficiency in the engineering of the glycosylation pathway, as illustrated below. However, there will also be limits to N-glycosylation engineering in plants. This is illustrated by our recent results demonstrating the importance of the early steps of N-glycan maturation for the accumulation of seed storage proteins, the formation of protein bodies and cell differentiation during plant embryo development (Boisson *et al.*, 2001).

Expression of mammalian glycosyltransferases in the plant Golgi apparatus

Another attractive strategy to humanize plant N-glycans is to express mammalian glycosyltransferases in the plant, which would complete and/or compete with the endogenous machinery of N-glycan maturation in the plant Golgi apparatus. Based on these complementation strategies, we have hypothesized recently that the expression of human β(1,4)-galactosyltransferase, in the Golgi of plant cells, could lead to a partial humanization of plant N-glycans and, possibly, compete with the addition of β(1,2)-xylose and α(1,3)-fucose. In agreement with this hypothesis, we have shown that the human β(1,4)-galactosyltransferase, when expressed in plant cells, transfers galactose residues on to the terminal N-acetylglucosamine residues of plant N-glycans. Moreover, 30% of N-glycans carried on an antibody, produced in tobacco plants expressing this human galactosyltransferase, bear terminal N-acetyllactosamine sequences of the mammalian type. (Figure 6) (Bakker *et al* , 2001). The efficiency of this strategy is further increased when a targeted expression of human β(1,4)-galactosyltransferase is obtained after fusion of the catalytic domain of this glycosyltransferase with the first 54 amino acids of *A. thaliana* β(1,2)-xylosyltransferase (Bakker *et al* , 2003).

However, as the N-glycans borne by the tobacco-derived antibodies are highly heterogeneous, the action of the human β(1,4)-galactosyltransferase on this pool of glycans resulted in a highly complex mixture of N-glycans, some of them being partially humanized (Bakker *et al.*, 2001). These strategies developed to glycoengineer plant-made antibodies would be more efficient in plant systems such as alfalfa, where the N-glycosylation of antibodies is restricted to a predominant mature oligosaccharide chain harbouring terminal GlcNAc residues. These glycans present the perfect structure for *in vitro* or *in vivo* remodelling into a human-compatible N-glycosylation As a proof of concept, we have shown that *in vitro* galactosylation using a β(1,4)-galactosyltransferase of an antibody-made in alfalfa resulted in an efficient conversion of the plant N-glycan into oligosaccharides having homogeneously galactosylated antennae identical to those of the murine antibody (Bardor *et al.*, 2003b).

Concluding remarks

The promising results already obtained for the humanization of N-glycosylation will hopefully permit the creation of plant systems producing PMPs compatible with human therapy in the near future. Current efforts are concentrated on the association of complementation strategies with strategies allowing the inhibition of plant glyco-epitope biosynthesis. Our next goal, in the near future, is to produce plant-made antibodies presenting an N-glycosylation profile identical to that observed in mammals. In this respect, key steps will include the inactivation of plant β(1,2)-xylosyltransferase and α(1,3)-fucosyltransferase using strategies that prove to be more efficient than the antisense oligonucleotide approach and improvement of the human β(1,4)-galactosyltransferase efficiency. We are currently working at increasing the performance of this heterologous glycosyltransferase through a better control of its targeting in the Golgi cisternae.

Compared with the other plasma proteins, antibodies present a simple N-glycosylation, with partial terminal β(1,4)-galactosylation and very few terminal sialic acids. However, sialic acids are very important for the clearance of many mammalian plasma proteins of pharmaceutical interest. The absence of such residues on these circulating proteins induces their fast elimination from the blood by interactions with galactose-specific receptors on the surface of hepatic cells. To our knowledge, sialic acids are absent from plant cells. Adapting the maturation machinery of plant N-glycans to obtain sialylated N-glycans in plants would require

the transfer of at least five heterologous genes encoding enzymes implicated in sialic acid biosynthesis, and transport within the Golgi. These enzymes should not only be expressed in a stable manner, but should also be active and correctly targeted in a plant cell.

Therefore, the production of humanized antibodies in plants pertains to today's technology, whereas the production of sialylated PMPs at large remains a much more ambitious objective for plant biotechnology.

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Appendix 5

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Transgenic Expression of Bean α-Amylase Inhibitor in Peas Results in Altered Structure and Immunogenicity

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The development of modern gene technologies allows for the expression of recombinant proteins in non-native hosts. Diversity in translational and post-translational modification pathways between species could potentially lead to discrete changes in the molecular architecture of the expressed protein and subsequent cellular function and antigenicity. Here, we show that transgenic expression of a plant protein (α-amylase inhibitor-1 from the common bean (*Phaseolus vulgaris* L. cv. Tendergreen)) in a non-native host (transgenic pea (*Pisum sativum* L.)) led to the synthesis of a structurally modified form of this inhibitor. Employing models of inflammation, we demonstrated in mice that consumption of the modified αAI and not the native form predisposed to antigen-specific $CD4^+$ Th_2-type inflammation. Furthermore, consumption of the modified αAI concurrently with other heterogeneous proteins promoted immunological cross priming, which then elicited specific immunoreactivity of these proteins. Thus, transgenic expression of non-native proteins in plants may lead to the synthesis of structural variants possessing altered immunogenicity.

KEYWORDS: α-Amylase inhibitor; transgenic plant; animal model; Th2 inflammation; mass spectrophotometry

INTRODUCTION

Genetically modified (GM) plants are designed to enhance agronomic productivity or product quality and are being increasingly employed in both agricultural and livestock industries (*1*, *2*). Recently, peas (*Pisum sativum* L.) expressing a gene for α-amylase inhibitor-1 (αAI) from the common bean (*Phaseolus* vulgaris L. cv. Tendergreen) were generated to protect the seeds from damage by inhibiting the α-amylase enzyme in old world bruchids (pea, cowpea, and azuki bean weevils) and are currently undergoing risk assessments (*3*–*6*).

The present study was initiated to (1) characterize the proteolytic processing and glycopeptide structures of αAI when transgenically expressed in peas (pea-αAI) and (2) evaluate in an in vivo model system the immunological consequence of oral consumption of pea-αAI. We demonstrate that expression of αAI in pea leads to a structurally modified form of this inhibitor. Employing experimental models, we show that the structural modification can lead to altered antigenicity. These investigations reveal that expression of proteins in non-native hosts can lead to the synthesis of a protein variant with altered immunogenicity.

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MATERIALS AND METHODS

Nontransgenic and Transgenic Plants. Seed meal was obtained from nontransgenic peas, genetically modified peas expressing bean α-amylase inhibitor-1 (αAI) (*5*), genetically modified narrow leaf lupin (*Lupinus angustifolius* L.) expressing sunflower seed albumin protein (SSA) in the seeds (SSA-lupin) (*7*), and from nontransgenic Pinto bean. Seeds were ground into fine flour in liquid N_2 using a mortar and pestle. This seed meal was then suspended in PBS (0.166 g meal/mL), homogenized, sieved through a 70 μm mesh, and stored at −70 °C. In some experiments, seed meal homogenates were cooked at 100 °C for 30 min before administration to mice (indicated in text).

Purification of SSA from Transgenic Lupin and αAI from Common Beans and from Transgenic Peas. αAI was purified from the common beans (Pinto and Tendergreen) and transgenic peas and SSA from genetically modified narrow leafed lupin (SSA-lupin) as previously described (*7*, *8*). Purified proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 15–

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25% gradient, 1 mm thick, mini-gel format) and MALDI-TOF mass spectrometry.

Western Immunoblot Analysis. αAI polypeptide composition was determined in protein extracts from common bean and transgenic peas as previously described (*3*). Protein was extracted from seeds with 0.5 M NaCl, 1 mM EDTA, and 0.1 M *N*-tris(hydroxymethyl)methylaminoethanesulfonic acid at pH 7.8. Aliquots of reduced protein (20 μg by Bradford assay) were fractionated by SDS-PAGE and electroblotted onto nitrocellulose membrane. αAI polypeptides were detected with an αAI antiserum from rabbit and goat anti-rabbit IgG conjugated to alkaline phosphatase (*3*). The concentration of αAI in transgenic peas was determined as 4% of total protein as previously described (*3*).

Structural Analysis of Purified αAI from the Pinto and Tendergreen Beans and from Transgenic Peas. Purified αAI from the common beans, Pinto and Tendergreen, and from transgenic peas were analyzed by matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS). The proteins were dissolved in water (approximately 1 μg/μL), and then 1 μL was mixed with 1 μL of matrix solution (saturated sinapinic acid in 50% acetonitrile/0.1% trifluoroacetic acid) on the sample plate of a Voyager Elite MALDI-TOF mass spectrometer (Perseptive Biosystems) and allowed to dry. Spectra were collected in linear mode with myoglobin used for close external calibration (Sigma, Cat. No. M-1882, 16952.6 $[M + H]^+$, 8476.8 $[M + 2H]^{2+}$).

Mice and Intragastric Administration of Seed Meal from Nontransgenic and Transgenic Plants. BALB/c mice were obtained from specific pathogen-free facilities at the Australian National University. Mice were intragastrically administered 250 μL of seed meal suspension (~100 mg/mL) containing either transgenic peas, nontransgenic peas, SSA-lupin, or Pinto bean twice a week for 4 weeks. In some experiments, serum was taken from the mice at the start of the third and fifth weeks during feeding. The serum antibody titers were determined as previously described (*9*).

Mice and Delayed Type Hypersensitivity Responses. BALB/c mice were administered seed meal as described above. Seven days following the final intra-gastric challenge, mice were subcutaneously injected with 25 μL of the appropriate antigen [Tendergreen-αAI, pea α-AI, or lupin SSA (1 mg/mL in PBS)] into the footpad. The positive control [(+) control] is mice immunized by i.p. injection of 200 μL containing 50 μg of Tendergreen-αAI dissolved in PBS with Alum (1 mg/mL) and subsequently receiving 25 μL of purified Tendergreen-αAI (1 mg/mL PBS). The negative control [(−) control] is mice immunized by i.p. injection of 200 μL containing 50 μg of Tendergreen-αAI dissolved in PBS with Alum (1 mg/mL) and subsequently receiving 25 μL of PBS. DTH responses were assessed by measuring the specific increase in footpad thickness using a digmatic calliper (Mitutoyo, Kawasaki, Japan) 24 h following the challenge. Serum was collected on day 14, and antibody titers were determined as previously described (*9*).

Murine Model of $CD4^+$ Th2 Cell-Mediated Inflammation. BALB/c WT mice were administered seed meal as indicated in the text. Seven and nine days following the final intra-gastric challenge, mice were anesthetized with an intravenous injection of 100 μL of Saffan solution (1:4 diluted in PBS). Mice were intubated with a 22 gauge catheter needle, through which purified αAI from Tendergreen bean or transgenic pea (1 mg/mL PBS), or vehicle control (PBS), was instilled. Airway responsiveness (AHR), mucus production, and eosinophilia were measured 24 h following the final intra-tracheal challenge. AHR to methacholine was assessed in conscious, unrestrained mice by barometric plethysmography, using apparatus and software supplied by Buxco (Troy, NY) as previously described (*9*). This system yields a dimensionless parameter known as enhanced pause (Penh), reflecting changes in waveform of the pressure signal from the plethysmography chamber combined with a timing comparison of early and late expiration, which can be used to empirically monitor airway function. Measurements were performed as previously described (*9*). Lung tissue representing the central (bronchi-bronchiole) and peripheral (alveoli) airways was fixed, processed, and stained with Alcian Blue-PAS for enumeration of mucin-secreting cells or Charbol's chromotrope-Haematoxylin for identification of eosinophils as previously described (*9*).

Intragastric Administration of Purified αAI and OVA. Mice were administered 200 μL of affinity purified Tendergreen- or transgenic pea-αAI (5 μg) with ovalbumin (OVA, 1 mg/mL) in a PBS suspension three times a week for 2 weeks. One week following feeding, the mice were intubated with a 22 gauge catheter needle, through which 25 μL of OVA (1 mg/mL PBS), or vehicle control (PBS), was instilled and the $CD4^+$ Th2-inflammation indices determined as described above. Serum was taken from the mice 1 day after the final intra-tracheal challenge, and serum antibody titers were determined as described (*9*).

Antigen Specific $CD4^+$ T-Cell Response. Peribronchial lymph nodes (PBLN) were subjected to pea-αAI or αCD3/αCD28 stimulation as previously described (*9*). In brief, 5×10^5 PBLN cells/mL were cultured with αAI (50 μg/mL) or αCD3 (5 μg/mL)/αCD28 (1 μg/mL) for 96 h. IL-4, IL-5, IFNγ levels were determined in supernatants from stimulated PBLN homogenates by using the OptEIA Mouse IL-4, IL-5, and IFNγ kits (Pharmingen).

Statistical Analysis. The significance of differences between experimental groups was analyzed using Student's unpaired *t*-test. Values are reported as the mean ± SEM. Differences in means were considered significant if $p < 0.05$.

RESULTS

MALDI-TOF-MS Analysis of αAI. To assess the consequences of transgenic expression of the bean αAI in peas, we initially performed a structural analysis of the transgenically expressed protein (pea-αAI). Pea-αAI was compared by Western blot analysis and MALDI-TOF-MS with natively expressed αAI from the common beans, cvs. Pinto (Pinto-αAI) and Tendergreen (Tendergreen-αAI) (collectively termed bean-αAI). Previous studies have shown that bean-αAI is synthesized as a pre-pro-αAI polypeptide that is cleaved following Asn^{77} to form two peptide chains (α and β), both of which are glycosylated and have one or more amino acid residue(s) removed from their C-termini (*8*). This post-translational processing results in major forms of the α and β chains with masses of 11 646 and 17 319, respectively, and minor forms containing alternative glycans (*10*−*12*). Western immunoblot analysis of Tendergreen-αAI and pea-αAI revealed immunoreactive bands in the 11 000−18 000 mass range consistent with the reported structure (*10*−*13*). Detailed comparison of Tendergreen-αAI with pea-αAI revealed differences in the banding profile, suggesting possible differences in the molecular structure of natively and transgenically expressed αaI (**Figure 1A**).

To better resolve the differences between pea-αAI and bean-αAI, affinity purified αAI was analyzed by MALDI-TOF-MS (**Figure 1B**). The mass spectra of Tendergreen-αAI and Pinto-αAI closely matched a previously published spectrum (*10*) of a bean-αAI (*Phaseolus vulgaris* L. cv. Greensleeves) confirming that both Tendergreen- and Pinto-αAI possess similar well-characterized post-translational modifications and very similar relative abundance of minor processing variants (*10*, *11*). Alignment of our spectra with the previously published data (*10*) allowed identification of peaks in the pea-, Tendergreen-, and Pinto-αAI spectra. The major form of the α-chain (11 646 Da) of bean-αAI contains residues 1−76 by cleavage of the pro-protein following Asn^{77}, removal of Asn^{77}, and the addition of sugar residues ($Man_6GlcNAc_2$ at Asn^{12} and $Man_9GlcNAc_2$ at Asn^{65}). Minor forms of the α-chain of bean-αAI differed by having one to three fewer mannose residues resulting in a series of peaks in the MALDI-TOF spectrum that differ by 162 mass units. In contrast, less heavily glycosylated forms dominated for the α-chain of pea-αAI. In particular, an α-chain with two fewer mannose residues (11 322 Da) was the most abundant for pea-αAI but the least abundant for Tendergreen-αAI (**Figure 1C(i)**). A further difference in the pea-αAI spectrum was a series of minor peaks differing from the main α-chain peaks by either

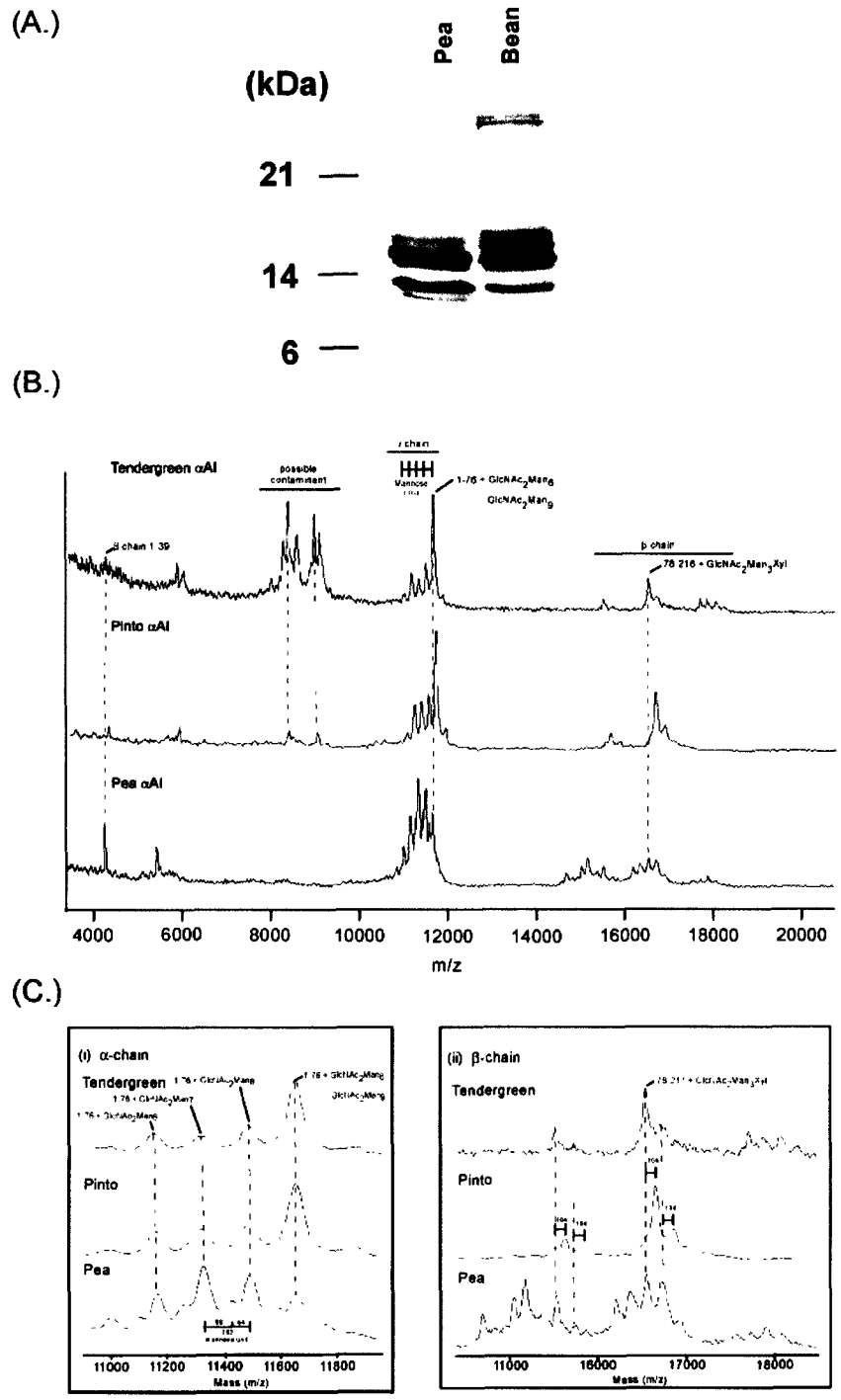

Figure 1. Western immunoblot and MALDI-TOF-MS analysis of common bean-derived-αAIs and αAI from transgenic peas. **(A)** Western blot analysis of αAI protein in extracts of transgenic peas and the Tendergreen variety of common bean. The masses of standard proteins are indicated. **(B)** Aligned MALDI-TOF mass spectra of purified αAI from transgenic pea and the common beans, Tendergreen and Pinto. **(C)** Detail from the spectra in panel B showing the regions of the α-chain (i) and the β-chain (ii).

+98 or −64 mass units, indicating another modification of some of the pea-αAI α-chains (**Figure 1C(i)**).

The major form of the β-chain of Greensleeves-αAI (16527 Da) contains residues 78−216 by cleavage of the pro-protein following Asn^{77}, the removal of the seven C-terminal residues following Asn^{216}, and the addition of sugar residues ($Man_3GlcNAc_2Xyl_1$ at Asn^{140}) (*10*−*13*). The β-chain region of the Tendergreen-αAI spectrum closely aligned with that of Greensleeves-αAI (**Figure 1C**). The β-chain region of the Pinto-αAI spectrum also closely resembled that of Greensleeves-αAI except that both major and minor peaks of Pinto-αAI were shifted by approximately +104 mass units. This mass discrepancy is consistent with five amino acid residue differences between the β-chains of Tendergreen-αAI and Pinto-αAI as predicted by gene sequence comparison (see Supporting Information Figure 1). Further, there are also three predicted residue differences between the Tendergreen-αAI and Pinto-αAI α-chains that result in a difference of +1 mass unit, which would not be

detected by our methods. These sequence differences are consistent with previous reports of αAI polymorphisms among bean cultivars (*12*, *13*). The pea-αAI spectrum showed major peaks corresponding to the two major and minor forms of the β-chain found in Tendergreen-αAI; however, the pea-αAI spectrum also showed a number of other peaks (**Figure 1C(ii)**). DNA sequencing of the transgene in pea and comparison with the published sequence (*14*) confirmed that the nucleotide sequences were identical, establishing that the observed further forms of the pea-αAI are related by variations in post-translational modifications including glycosylation (**Figure 1C(ii)**).

Analysis of the spectra of pea- and bean-αAI also revealed several other differences. First, a number of peaks at ~8−9000 and 5824 mass units and below were observed in the bean-αAI spectrum, which are consistent with a previously reported protein that copurifies with bean-αAI (*10*) and doubly charged ($(MH_2)^{2+}$) forms of the α-chain, respectively. Further, a peak at 4223 mass units was detected in the pea-αAI spectrum, which has not been previously reported. While this peak is barely detected in the bean-αAI spectrum presented here, the peak was observed in a number of other bean-αAI preparations (results not shown). The mass of this peak is consistent with the first 39 residues of the β-chain, which could be obtained by cleavage following an Asn residue, the same protease specificity that provides the reported processing of αAI at Asn^{77}. Consistent with this hypothesis, a small peak was detected in some preparations at about 12 304 mass units that could correspond to the remainder of the β-chain.

While pea-αAI has not yet been characterized as thoroughly as the bean-αAI, it is clear that the transgenic expression of the bean αAI gene in the pea led to differences of glycosylation and possibly other differences in both the α- and the β-chains.

Immunological Consequence of Oral Consumption of Beans. Peas are used as a feed component in the livestock industry and also in human diets. Generally, dietary protein antigens undergo gastric digestion leading to the formation of nonimmunogenic peptides and the induction of a state of specific immunological unresponsiveness termed oral tolerance (*15*, *16*). However, the demonstration of structural differences between the transgenic αAI in pea and the natively expressed bean forms raised the concern that the tolerance mechanism may be perturbed, possibly leading to enhanced immunoreactivity.

The induction of oral tolerance results in the failure of the immune system to elicit an active immune response to subsequent exposure to the same antigen in the skin (delayed type hypersensitivity [DTH] response) or lung ($CD4^+$ T-helper [Th_2] cell-mediated inflammation). To examine potential differences in immunological responsiveness following oral consumption, mice were fed Pinto bean, which expresses a native form of αAI and subsequently received purified Tendergreen-αAI in the skin and lung. Most varieties of common beans such as Red Kidney or Tendergreen contain high levels of phytohemagglutinin (PHA), an anti-nutritional factor that induces dietary toxicity in rodents and birds. We therefore used the Pinto variety that contains very low levels of PHA (*17*, *18*) as the appropriate control for oral exposure. Oral consumption of native uncooked Pinto bean seed flour followed by intra-tracheal (i.t.) challenge with Tendergreen-αAI or phosphate buffered saline (PBS) failed to induce an αAI-specific IgG_1 antibody response (**Figure 2A**). Similarly, sub-cutaneous (s.c.) challenge of the footpad or i.t. challenge of Pinto bean-fed mice with Tendergreen-αAI also failed to promote a DTH response (results not shown) or a pulmonary Th_2-inflammatory response [pulmonary eosinophilia, mucus hypersecretion, and enhanced AHR to a bronchoconstrictive agents], respectively (**Figure 2B−D**). While the level of AHR in the Pinto bean-fed αAI-challenged mice was higher than PBS-challenged mice, the level of responsiveness is not significantly different from that of naïve mice i.t. challenged with Tendergreen-αAI (**Figure 2D**). As a positive control, mice were sensitized by intra-peritoneal (i.p.) injection and subsequently challenged via the airways with bean-derived αAI to induce immunological responsiveness (**Figure 2A−D**). Collectively, these data showed that oral consumption of the native bean form of αAI followed by respiratory exposure to bean-αAI did not promote immunological responsiveness or inflammation.

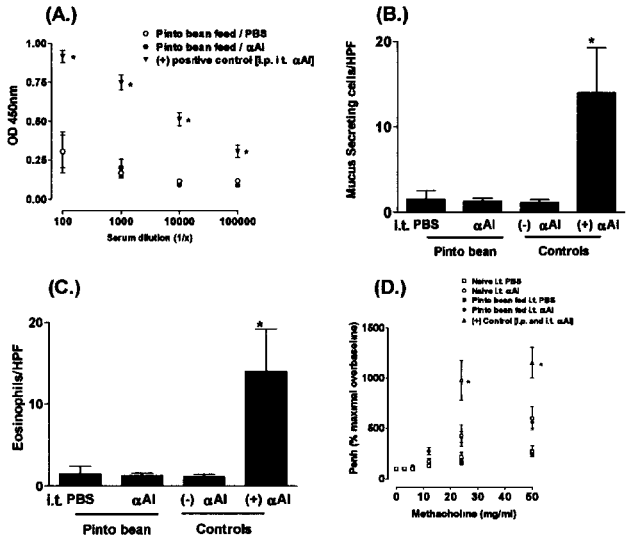

Figure 2. Experimental consumption of bean (cv. Pinto) seed meal does not predispose to inflammation. (**A**) αAI-specific IgG_1 in serum and (**B**) mucus-secreting cell numbers and (**C**) eosinophil levels in lung tissue from Pinto bean-fed mice i.t. challenged with PBS or Tendergreen-αAI. (**D**) AHR in Pinto bean-fed mice i.t. challenged with PBS or Tendergreen-αAI. Data are expressed as the (**A–D** and **F**) mean ± SEM and (**E**) mean O.D. of the serum dilution 1/10 ± SEM from 4 to 6 mice per group from duplicate experiments. (**A–D**) * $p < 0.05$ as compared to Pinto bean-fed i.t. αAI.

Immunological Consequence of Oral Consumption of Transgenic Peas. To determine whether oral consumption of the transgenic αAI (from pea) elicited an immunological response, mice were orally administered transgenic pea seed meal and αAI; serum antibody titers and DTH responses were examined. Interestingly, in mice that were fed transgenic pea, but not nontransgenic pea, αAI-specific IgG_1 was detected at 2 weeks and at significant levels after 4 weeks of oral exposure (**Figure 3A**). Consistent with the antibody findings, mice fed nontransgenic pea seed meal did not develop DTH responses following footpad challenge with purified pea-αAI (**Figure 3B**). In contrast, mice fed transgenic pea seed meal exhibited a significant DTH response as compared to the nontransgenic pea exposed group when purified pea-αAI was injected into the footpad (**Figure 3B**). As a control for any general effect of genetic modification, we repeated the experiment with material from two other genetically modified plants, lupin (*Lupinus angustifolius* L.) expressing sunflower seed albumin (SSA) [transgenic lupin] (*9*) and chickpeas (*Cicer arietinum* L.) expressing bean derived αAI. Mice were orally administered lupin or transgenic lupin or chickpea or transgenic chickpea seed meal and subsequently footpad challenged with SSA or αAI and DTH responses were examined. In contrast to transgenic pea, mice fed transgenic lupin or chickpea did not develop

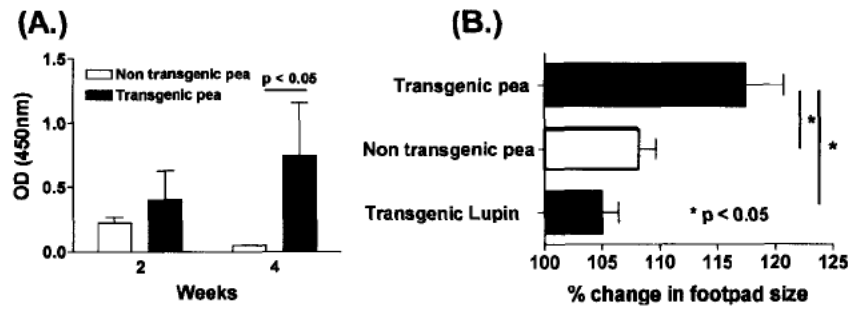

Figure 3. Experimental consumption of transgenic pea seed meal predisposed to antigen-specific IgG_1 and DTH responses. (**A**) Antigen-specific IgG_1 and (**B**) DTH responses in pea nontransgenic and pea transgenic-fed mice. Data are expressed as the (**F**) mean ± SEM and (**E**) mean O.D. of the serum dilution 1/10 ± SEM from 4 to 6 mice per group from duplicate experiments. (**A–C**) * $p < 0.05$ as compared to nontransgenic pea or transgenic lupin fed mice i.t. αAI.

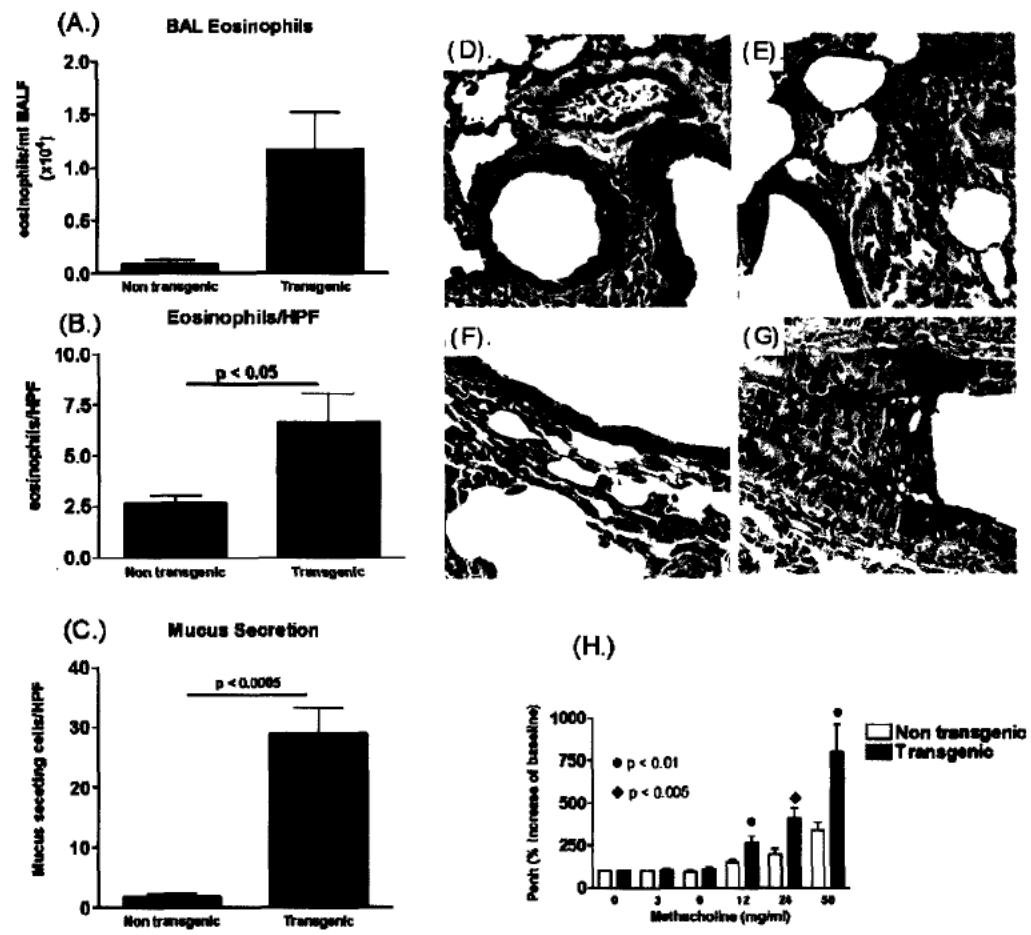

Figure 4. Consumption of transgenic pea seed meal predisposed to $CD4^+$ Th_2-type inflammatory response. Eosinophil accumulation in bronchoaveolar lavage fluid (BAL) (**A**), tissue (**B**), and mucus-secreting cell numbers (**C**) in lung tissue from nontransgenic and transgenic pea-fed mice i.t. challenged with αAI purified from pea. (**D–G**) Representative photomicrographs of eosinophil accumulation in lung of (**D**) nontransgenic and (**E**) pea transgenic-fed mice and mucus-secreting cell numbers in lung tissue of (**F**) nontransgenic and (**G**) pea transgenic-fed mice i.t. challenged with αAI from pea. (**H**) Airways hyperresponsiveness (AHR) in nontransgenic and pea transgenic-fed mice i.t. challenged with αAI from pea. Data are expressed as the mean ± SEM from 3 to 6 mice per group from duplicate experiments. Statistical significance of differences ($p < 0.05$) was determined using Student's unpaired *t*-test. (**D–G**) ×400 magnification.

DTH responses following footpad challenge with the transgenically expressed and purified SSA or αAI protein (**Figure 3B**; results not shown). Thus, consumption of transgenic pea containing αAI promoted αAI-specific immunological responsiveness.

To characterize the type of immune response elicited against pea-αAI following oral consumption of transgenic pea, we employed a well-characterized murine model of $CD4^+$ Th_2 cell-mediated inflammation (*19*). Mice were orally administered transgenic pea seed meal and subsequently i.t. challenged with purified pea-αAI, and key features of Th_2-inflammation [pulmonary eosinophilia, mucus hypersecretion, and AHR] were examined. I.t. challenge of nontransgenic pea-fed mice with purified pea-αAI failed to induce features of Th_2-inflammation (**Figure 4A–G**). Furthermore, airways responsiveness to the cholinergic spasmogen, methacholine, was not induced in these

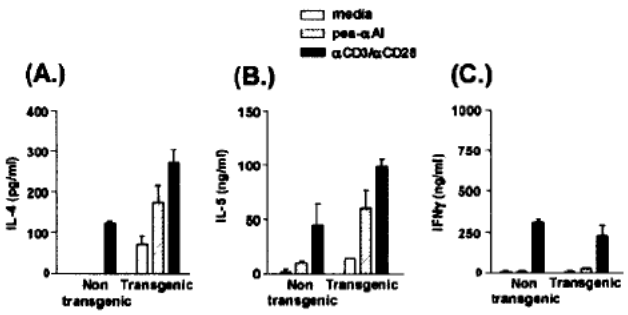

Figure 5. Consumption of transgenic pea seed meal predisposed to $CD4^+$ T-cell derived Th_2-type cytokine production. IL-4 (**A**), IL-5 (**B**), and IFNγ (**C**) levels in supernatants from αCD3/αCD28 or pea-αAI or media alone stimulated PBLN cells from nontransgenic and transgenic pea-fed mice i.t. challenged with αAI from pea. Data are expressed as the mean ± SEM from 3 to 6 mice per group from duplicate experiments. Statistical significance of differences ($p < 0.05$) was determined using Student's unpaired *t*-test.

mice (**Figure 4H**). However, instillation of pea-αAI into the lungs of mice fed transgenic pea induced key features of Th_2-type inflammation including pulmonary eosinophilia, mucus hypersecretion, and AHR (**Figure 4A−H**).

Pulmonary eosinophilia, mucus hypersecretion, and AHR are critically linked to the effector function of the Th_2 cytokines (*20*). To examine whether consumption of transgenic pea promoted a αAI-specific $CD4^+$ Th_2-type T-cell response, $CD4^+$ T-cells in peribronchial lymph node (PBLN) cultures from mice fed nontransgenic pea or transgenic pea seeds challenged with pea-αAI were stimulated with pea-αAI and cytokine profiles determined. Stimulation of $CD4^+$ T-cells in peribronchial lymph node (PBLN) cultures from nontransgenic pea-fed mice challenged with pea-αAI did not elicit Th_2 (interleukin (IL)-4 and IL-5)- or Th_1-type (gamma interferon, IFNγ) cytokine production in response to pea-αAI stimulation (**Figure 5A−C**). By contrast, stimulation of PBLN cultures with pea-αAI from i.t. challenged mice fed transgenic pea resulted in the significant production of Th_2 cytokines (**Figure 5A−C**). Thus, oral exposure of mice to transgenic pea, but not nontransgenic seed meal, predisposed to systemic immunological responsiveness characterized by a Th_2-type immune profile.

Pea-αAI Promotes Immune Responses to Other Oral Antigens. Previous investigations have demonstrated that various plant-derived proteins such as tomatine possess immunomodulatory activity and potentiate and polarize immune responses (*21−23*). We have demonstrated that consumption of transgenic pea in the presence of a large number of potential dietary antigens in the gastrointestinal tract induces an active systemic Th_2-immune response against pea-αAI. In light of these findings, we were next interested in determining whether consumed pea-αAI possessed immunomodulatory activity for Th_2 immune responses and could sensitize mice to heterogeneous nongenetically modified food antigens. Thus, we intragastrically (i.g.) administered purified Tendergreen- or pea-αAI with the well-characterized dietary antigen, chicken egg white protein OVA, or OVA alone and subsequently i.t. challenged mice with OVA. I.g. administration of OVA alone did not systemically sensitize mice to OVA (**Figure 6A**). Further, subsequent OVA challenge in the airways did not promote Th_2-inflammation (mucus hypersecretion, pulmonary eosinophilia, or AHR). Similarly, i.g. administration of bean-αAI and OVA did not systemically sensitize mice or predispose to Th_2-inflammatory processes. However, consumption of pea-αAI and OVA promoted a strong OVA-specific Th_2-type antibody

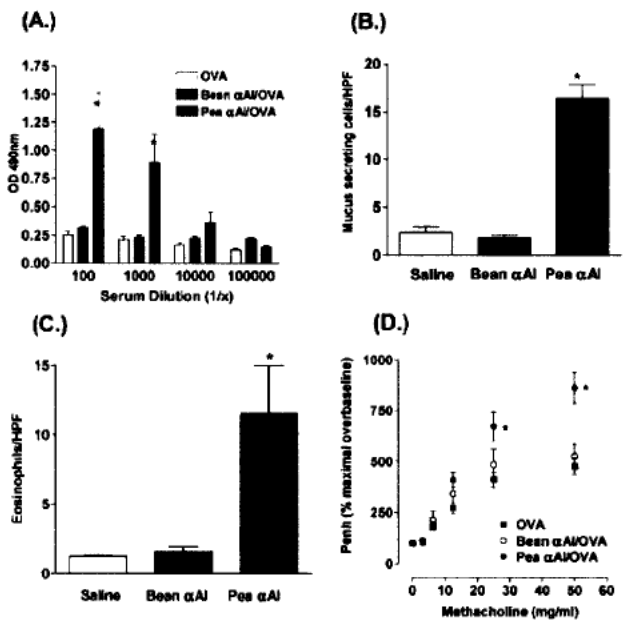

Figure 6. Intra-gastric administration of αAI from pea induces cross-priming of heterogeneous food antigens. OVA-specific IgG_1 levels (**A**) and the Th_2-inflammation phenotype (mucus hypersecretion) (**B**), pulmonary eosinophilia (**C**), and airways hyperreactivity (**D**) in mice that were fed (i.g. challenged) ovalbumin (OVA) alone (the control) or in combination with natively expressed Tendergreen bean-αAI or transgenically expressed (pea) αAI and subsequently intra-tracheal challenged with purified OVA. Data are expressed as the mean ± SEM from 4 to 6 mice per group. * $p < 0.05$ as compared to OVA and bean αAI/OVA.

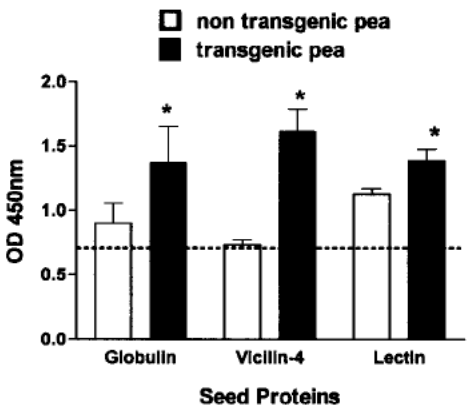

Figure 7. αAI from pea induces cross-priming of pea proteins. Pea globulin-, vicilin-4, and lectin-specific IgG_1 levels in serum from mice that were intragastrically administered 250 μL (~100 mg/mL) of either nontransgenic or transgenic pea seed meal twice a week for 4 weeks. Data are expressed as mean ± SEM from 4 to 5 mice per group. * $p < 0.05$ as compared to nontransgenic pea.

response (**Figure 6A**) and predisposed mice to OVA-induced Th_2-inflammation (**Figure 6B−D**). To support this observation, we examined serum levels of antigen-specific IgG_1 against pea seed proteins (pea globulins, lectin, and vicilin-4) in transgenic pea and nontransgenic pea-fed mice. Interestingly, levels of antigen-specific IgG_1 against pea globulins, lectin, and vicilin-4 in serum of transgenic pea fed mice were significantly higher than those of nontransgenic pea-fed mice, suggesting a heightened immune responsiveness to dietary proteins due to pea-αAI (**Figure 7**). Thus, these studies demonstrate that modified αAI possesses immunodulatory activity and that consumption

of the modified αAI concurrently with heterogeneous proteins can promote immunological cross priming, which predisposes to specific immunoreactivity to these proteins.

DISCUSSION

Recently, peas expressing a gene for αAI from the common bean were generated for protection against field and storage pests (*3*−*6*). Characterization of αAI by structural analysis has demonstrated that transgenic expression of this protein in peas led to the synthesis of a modified form of αAI. Further, we show that the modified form of αAI possessed altered antigenic properties and consumption of this protein by mice predisposed to αAI-specific $CD4^+$ Th_2-type inflammation and elicited immunoreactivity to concurrently consumed heterogeneous food antigens.

Bean-αAI undergoes significant post-translational modification including variable glycosylation and proteolytic processing leading to the synthesis of a mature functional protein (*8*, *11*). We demonstrate that differences in glycosylation and/or other modifications of the pea-αAI lead to altered antigenicity. Consistent with our observations, investigators have previously demonstrated that differential glycosylation of subunits of a cereal α-amylase-inhibitor family (unrelated to legume αAIs) enhances IgE-binding capacity (*24*). Moreover, glycosylated cereal αAI subunits have been shown to possess significantly enhanced IgE-binding affinity when compared to the unglycosylated forms (*24*). These cereal proteins possess identical amino acid sequences and only differ in their carbohydrate moieties, indicating that glycosylation can confer IgE-binding capacity and Th_2-inflammation. In particular, recent investigations have demonstrated that glycan side chains linked to high mannose-type N-glycans on plant-derived glycoproteins can confer immunogenicity and are IgE binding determinants (*25*, *26*). Moreover, α(1,3)-fucose and β(1,2)-xylose linkage to high mannose-type N-glycans ($Man_5GlcNAc_2$−$Man_9GlcNAc_2$) promote immunogenicity and IgE binding. The β-chain of pea-αAI possesses β(1,2)-xylose linked high mannose-type N-glycans, and other complex glycoforms and the α-chain may possess an as yet undefined glycoform variant, and it remains to be determined how these modifications alter pea-αAI immunogenicity.

Functional and structural properties of pea-αAI may contribute to its ability to circumvent immune tolerance and elicit inflammatory responses. Bean-αAI is a potent inhibitor of human α-amylase activity and can induce gastrointestinal dysfunction (*27*). Comparison of bean- and pea-derived αAI activity revealed no difference in enzymatic activity between the two proteins (results not shown). Furthermore, we examined the gastrointestinal tract of pea and transgenic pea-fed mice and observed no histological abnormalities to the gastrointestinal tissue in either group (results not shown). Bean-αAI is also a heat-stable protein and partially resistant to proteolytic degradation (*28*, *29*). Extensive boiling (100 °C for 20 min), while significantly reducing α-amylase inhibitory activity, failed to alter the ability of the transgenic pea to prime for Th_2-inflammation when challenged in the lung [results not shown: see Supporting Information Figure 2]. These findings are consistent with previous demonstrations that cooking of plant material such as lentils and peanuts does not diminish the allergenic potential of certain proteins (*30*, *31*). Furthermore, these studies suggest that the altered immunogenicity of αAI is unrelated to its properties as an amylase inhibitor.

We demonstrate that the immune response elicited against pea-αAI following oral consumption of transgenic pea is characterized by $CD4^+$ Th_2 cell-mediated inflammation, in particular, the presence of IL-4 and IL-5. To examine whether the immune response was dependent on IL-5 and eosinophils, we employed IL-5 and eotaxin-deficient mice. IL-5/eotaxin-deficient mice were i.g. administered nontransgenic and transgenic seed meal and subsequently i.t. challenged with purified αAI. We show that i.t. challenge of transgenic pea fed IL-5/eotaxin-deficient mice induced Th_2-inflammation that was significantly elevated over nontransgenic fed mice (*32*). These investigations suggest that the immune response elicited against pea-αAI following oral consumption of transgenic pea is not dependent on IL-5 and eosinophils.

In this study, we have demonstrated that transgenic expression of αAI in a pea can lead to the synthesis of a modified form of the protein with altered antigenic properties. Furthermore, we show that concomitant exposure of the gastrointestinal tract to modified αAI and heterogeneous food antigens cross primes and elicits immunogenicity. Currently, we do not know the frequency at which alterations in structure and immunogenicity of transgenically expressed proteins occur or whether this is unique to transgenically expressed αAI. These investigations, however, demonstrate that transgenic expression of non-native proteins in plants may lead to the synthesis of structural variants with altered immunogenicity.

ABBREVIATIONS USED

αAI, α-amylase inhibitor-1; pea (*Pisum sativum* L.), transgenic pea; *Phaseolus vulgaris* L. cv. Tendergreen, *Pisum sativum* L. expressing α-amylase inhibitor-1 from the common bean; MALDI-TOF-MS, matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry.

ACKNOWLEDGMENT

We thank Aulikki Koskinen and Anne Prins for excellent technical assistance and David Tremethick, Ian Young, and Klaus Matthaei for their helpful discussions and preparation of the manuscript. GenBank accession number for common bean cv. Pinto is AY603476.

Supporting Information Available: Amino acid sequence of αA1 from common bean and consumption of pea seed meal predisposed to Th_2-type inflammation. This material is available free of charge via the Internet at http://pubs.acs.org.

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JF050594V

Merker, Robert I

From: Nalubola, Ritu
Sent: Monday, May 02, 2005 2:30 PM
To: Merker, Robert I
Cc: June, Geraldine A; Satchell, Felicia B
Subject: FW: OFAS Question

Hi Bob,

Yes, that paragraph is fine with us.

Thanks,
Ritu

-----Original Message-----
From: Merker, Robert I
Sent: Friday, April 29, 2005 4:18 PM
To: Nalubola, Ritu
Cc: June, Geraldine A
Subject: RE: OFAS Question

Ritu,

So, if I used the following of our standard paragraphs, it would not give you heartburn?

Our use of "lactoferrin (human) purified from rice" in this letter should not be considered an endorsement or recommendation of that term as an appropriate common or usual name for the purpose of declaring the substance in the ingredient statement of foods containing that ingredient. 21 CFR 101.4 states that all ingredients must be declared by their common or usual name. In addition, 21 CFR 102.5 outlines general principles to use when establishing common or usual names for nonstandardized foods. Issues associated with labeling and the appropriate common or usual name of a food are the responsibility of the Office of Nutritional Products, Labeling, and Dietary Supplements.

Bob

-----Original Message-----
From: Nalubola, Ritu
Sent: Friday, April 29, 2005 1:22 PM
To: Merker, Robert I
Cc: June, Geraldine A; Kane, Rhonda R.
Subject: FW: OFAS Question

Hi Bob,

With regard to your question about source labeling, we have reviewed the relevant sections (which you identified) in the GRAS notice and don't see a concern with the source being included as part of the name of the ingredient. Based on the information about the ingredient in view of rice allergenicity, we would not object to the notifier's suggestion to appropriately declare the source in the name of this ingredient. Whether we would require it is another question and we did not address that at this time.

Thanks,
Ritu

-----Original Message-----
From: Merker, Robert I
Sent: Thursday, April 14, 2005 3:42 PM
To: Nalubola, Ritu
Subject: RE: OFAS Question

The source labeling comments are on pages 57 and repeated on page 71 - I highlighted them in yellow.

Bob

-----Original Message-----
From: Nalubola, Ritu
Sent: Thursday, April 14, 2005 3:39 PM
To: Merker, Robert I
Subject: RE: OFAS Question

Bob,

An electronic copy would be great. Thank you!

Ritu

-----Original Message-----
From: Merker, Robert I
Sent: Thursday, April 14, 2005 3:29 PM
To: Nalubola, Ritu
Cc: Kane, Rhonda R.; June, Geraldine A
Subject: RE: OFAS Question

Thanks, Ritu,

OK - do you mind an electronic copy or do you want paper? You'll note that the name below was ours - the company called it "recombinant human lactoferrin produced in rice." We didn't care for that name, because although the gene was introduced into rice via recombinant DNA technology, the protein isn't itself recombinant. For further complication, the gene actually is synthetic, though the protein has the amino acid composition of human lactoferrin.

I can mark up or flag the electronic copy to flag the information about source labeling (which was done in their section on allergenicity?)

Bob

-----Original Message-----
From: Nalubola, Ritu
Sent: Thursday, April 14, 2005 3:10 PM
To: Merker, Robert I
Cc: Kane, Rhonda R.; June, Geraldine A
Subject: RE: OFAS Question

Hi Bob,

Geraldine June, my team leader, passed on your email below to me for follow-up.

We would require source labeling only under specific circumstances; however, if the notifier proposed a name that includes the source, we may not object to it provided it is truthful and non-misleading. I see this substance listed as “lactoferrin (human) purified from rice” on OFAS’ GRAS website. Is that the name the notifier proposed? If you send me a copy of the GRAS notice, I can get some background on this substance and see

the notifier's statements related to source labeling. I will then discuss with folks here and get back to you on whether and what may be included about labeling in OFAS' response. Sounds OK?

Thanks,
Ritu

-----Original Message-----
From: Merker, Robert I
Sent: Tuesday, April 12, 2005 5:29 PM
To: Kane, Rhonda R.
Subject: question

Rhonda,

Sorry to put you on the spot. We're in kind of a funny place on our GRAS notice, GRN 162. This is a biotech product - human lactoferrin produced in rice. Though rice isn't a major allergen, the notifier has suggested source labeling for those who might react to rice, because there are residual rice proteins in the product and because the lactoferrin itself is glycosylated with plant-derived glycans, which in a very few individuals could be allergenic.

The question is - can or should we say ANYTHING about labeling (I'd rather not beyond "the notifier has suggested that source labeling will/could be used to inform those who might be allergic to rice") - the letter will have a section on "potential for allergencity," so this will be a rather confused and muddy area???

Bob

Robert I. Merker, Ph.D.
Food and Drug Administration
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FD

MEMORANDUM OF TELEPHONE CALL
Between
Delia Bethell, Ph.D., Ventria Biosciences
and
Robert I. Merker, Ph.D., Office of Food Additive Safety

Date: July 28, 2005
Time: 3:40 p.m.
Subject: Rehydration formula use levels in GRN 162 and GRN 174. How would levels apply to other medical foods uses.

Dr. Merker told Dr. Bethell that Ventria's description in GRN 162 of levels in medical foods were specific to oral rehydration fluids and requested clarification on how these levels would correspond to levels consumed for other medical foods uses that might not be fluids and whether Ventria desired to include such uses in its submission. Dr. Merker mentioned that these issues would also apply to GRN 174 on lysozyme.

Robert I Merker
Robert I. Merker, Ph.D.

cc: GRN 162, GRN 174
R/D: HFS-255: RIMerker: 8/8/05
F/T: HFS-255: RIMerker: 8/8/05

Merker, Robert I

From: Merker, Robert I

Sent: Wednesday, March 15, 2006 8:12 AM

To: 'delia bethell'

Cc: Fasano, Jeremiah; Mihalov, Jeremy J.; Twaroski, Timothy P; Dinovi, Michael J; Chanderbhan, Ronald F; Luccioli, Stefano; Jones, Kathleen; Flamm, Eric; Mattia, Antonia; Martin, Robert L

Subject: FDA's primary concern regarding lactoferrin.

Dr. Bethell:

This message is a follow-up to our meeting of February 1, 2006 in which we described our concerns about lactoferrin consumption to you and Diane McColl. Please forgive our delay in providing these comments.

Our largest concerns (but not our sole concerns), rest on the question whether the consumption of large amounts of recombinant human lactoferrin would result in the possibility of a breakdown of tolerance (an autoimmune Th1 response, non-IgE-based). Part of this concern rests on whether the antigenic epitopes resulting from plant-based glycosylation could lead to removal of active suppression of existing lactoferrin-reactive T and B cells. Is there published data on other products that would address the consumption of other human (or relevant model animal) N-linked glycoproteins produced in plants that have been consumed by humans (or those animals) at high levels?

Your notice discussed findings that autoantibodies to lactoferrin dismissed concerns about the presence of these antibodies because there was no connection to pathology in those instances in which anti-lactoferrin have been found in individuals with autoimmune diseases. We note that these autoantibodies were produced in response to presumably low levels of endogenous lactoferrin in patients that had already had autoimmune responses. Consumption of large doses of lactoferrin, particularly lactoferrin that differs slightly from the endogenous form with respect to small sequence or conformational differences, could theoretically act as a trigger to autoimmune responses for those genetically predisposed.

References below may provide a starting point for discussion with your GRAS panel members. In several publications, consumption of lactoferrin leads to increased levels of immunostimulatory molecules such as lymphokines or cytokines, and other publications report that production of these molecules or systemic exposure to lactoferrin may result in autoimmune responses.

One publication indicating a potential adverse effect of lactoferrin is:

Guillen, et al., J. Immunol. 2002: 168:3950-3957. Enhanced Th1 response to *Staphylococcus aureus* Infection in Human Lactoferrin-Transgenic mice.

Those pointing to increased levels of immunostimulatory molecules upon administration of lactoferrin include:

Hayes, al., 2005: Investigational New Drugs: 2005 (e-pub)

Sfeir et al., J Nutr: 134: 403–409, 2004.

Gracie, et al, J. Clin Invest 1999. **104:** 1393-14401 and a commentary on this article on pages 1337-1339.

Moreover, there simply is insufficient data on how long-term consumption of gram quantities of lactoferrin affects the general population, and some evidence to suggest that elevated lactoferrin exposure may cause adverse, pro-inflammatory effects.

Let's talk at your convenience regarding how best to engage your GRAS panel on these concerns. Once these concerns have been adequately addressed we can proceed to other issues.

Thanks for your patience while we try to work together to address these concerns.

Sincerely yours,

Bob Merker

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May 19, 2005

VIA HAND DELIVERY

Robert Martin, Ph.D. (HFS-255)
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
University Station
4300 River Road
College Park MD 20740-3835

Dear Dr. Martin:

We have become aware that a GRAS notice, Number 162, has been submitted for human lactoferrin purified from rice. In assessing the adequacy of this notice, we request that the Division of Biotechnology and GRAS Notice Review consider the comments in the enclosed letter. This letter was sent by us to officials in CDER and CFSAN in anticipation of the submission of a GRAS notice for recombinant human lactoferrin; we were not aware at the time that the notice had already been filed.

Per our telephone conversation this morning, please forward the attached letter to all relevant persons within the Division. Please do not hesitate to call me or Peter Barton Hutt at (202) 662-5522 if we can provide additional assistance.

Regards,

Ruth K. Miller

Enclosure

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February 4, 2005

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Re: Use of Recombinant Human Lactoferrin
In Food and Dietary Supplements

Dear Drs. Tarantino, Schneeman, Weiss, and Walton,

We have become aware that Ventria BioScience (Ventria) is conducting human clinical trials of recombinant human lactoferrin (rhLF) in the United States and that it intends to market its rhLF as an ingredient in medical food or dietary supplements.[1] In addition, Pharming Group

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[1] Celia Lamb, *Regulators Block Plans for Genetically Altered Rice*, Sacramento Business Journal, April 9, 2004 (Attachment 1); Celia Lamb, *Altered Rice Still Headed to Market*, Sacramento Business Journal, April 16, 2004 (Attachment 2).

N.V. (Pharming) recently announced its intention to file a notification with FDA that its rhLF product is generally recognized as safe (GRAS) for use in food.[2]

Recombinant human LF is not now, and has not ever been, used as an ingredient in food or dietary supplements, and the safety of such product as a food additive or new dietary ingredient has not been established. Our client, Agennix, Inc. (Agennix), has conducted substantial clinical testing of rhLF as a drug under investigational new drug (IND) applications prior to any entry of rhLF into the marketplace as an ingredient in a dietary supplement. This ingredient is therefore excluded from the definition of a dietary supplement, and any rhLF product marketed as a dietary supplement is an unapproved new drug in violation of section 505 of the Federal Food, Drug, and Cosmetic Act (FD&C Act).[3]

It is important that CFSAN and CDER coordinate regulation of the safety of rhLF in food, dietary supplements, and drugs. Whatever safety requirements are appropriate for drug uses of rhLF should also be applied to its food and dietary supplement uses.

We urge FDA to investigate the attached press reports and to take action to prevent the unlawful marketing of these products. At this time, rhLF can be classified only as an investigational new drug or an unapproved food additive in the United States.

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[2] Press Release, Pharming Announces Positive Results of Study with Human Lactoferrin (November 24, 2004), *available at* http://www.pharming.com/index.php?act=show&pg=90 (Attachment 3).

[3] Section 201(ff)(3) of the FD&C Act.

I. Background

In September 1996, FDA approved the initial IND submitted by Agennix for the study of rhLF in treating gastrointestinal disorders.[4] Since that time, FDA has approved five additional IND applications for the study of rhLF for use in treating dermal concerns,[5] asthma,[6] GVHD,[7] cancer,[8] and most recently, mucositis.[9] Agennix currently is conducting Phase II human clinical trials of rhLF for the treatment of cancer, asthma, and diabetic wounds.

Ventria is now also producing rhLF. According to press coverage, Ventria has been selling its rhLF "for research uses" since the fourth quarter of 2003.[10] Newspapers report that the company has begun clinical trials in Southern California of a product containing purified lactoferrin and iron for the treatment of iron deficiency.[11]

Pharming announced in November 2004 that an animal toxicology study of its rhLF showed positive results and would be published in a scientific journal.[12] Pharming also announced that it would submit the results of this and other studies, as well as expert opinions, to FDA in support of GRAS recognition of rhLF for use in food.

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4 IND No. 6799.

5 IND No. 8546.

6 IND No. 10897.

7 IND No. 11230.

8 IND No. 11728.

9 IND No. 11870.

10 *Altered Rice Still Headed to Market*, *supra* (Attachment 2).

11 *Id.*

12 Pharming Announces Positive Results of Study with Human Lactoferrin, *supra* (Attachment 3).

II. Recombinant hLF Differs From Natural hLF

Although human lactoferrin (hLF) naturally is present in breast milk, recombinant human lactoferrin (rhLF) may differ from natural hLF in significant ways. Ventria acknowledges that its rice-based rhLF differs from natural hLF in that "Three major types of glycans are present in recombinant human lactoferrin, and all of them carry the typical core structure of the plant glycan with both xylose and fucose. None carries sialic acid as in the native form of human lactoferrin."[13] Ventria acknowledges that "certain protein targets require human glycans for optimal efficacy and stability when reintroduced into the human system," and that recent commentary has discussed the importance of correct glycan structure.[14] In response, Ventria offers but does not support the assertion that "There should generally be little effect of plant glycan structures in plant produced proteins."[15]

FDA has long expressed concern regarding the potential differences between natural products and their recombinant counterparts, and required appropriate oversight of such recombinant agents.[16] Although some products derived through recombinant means have been approved for use, including recombinant Factors VIII and Factor VIIa and etanercept (Enbrel), these products have been carefully reviewed by FDA before approval. In requiring the IND applications submitted by Agennix, CDER has recognized the possibility that the recombinant

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13 Ning Huang, *High-Level Protein Expression System Uses Self-Pollinating Crops as Hosts*, BioProcess International 54, 55 (January 2004)(Attachment 4).

14 *Id.*

15 *Id.*

16 51 Fed. Reg. 23309 (June 26, 1986).

nature of the product may result in changes to its safety profile, and that these potential changes must be reviewed by FDA before the product may be tested or marketed.

III. RhLF Is Not GRAS for Use in Food and Would Require a Food Additive Petition and Regulation

Pharming has announced its intention to bring rhLF to the market as an ingredient in a medical food product. Ventria also has suggested that it may make rhLF available as a food ingredient. Neither of these companies legally can market rhLF as a component of food until FDA reviews a food additive petition and promulgates a food additive regulation.

The only pertinent exception to the requirement of a food additive regulation would be a GRAS determination. Absent a food additive regulation, Pharming and Ventria would be required to demonstrate that rhLF is GRAS prior to using it as a food ingredient, whether in traditional food or in medical food.[17] RhLF is not listed as GRAS in any FDA regulation and has not been the subject of a GRAS notification. Moreover, we find no evidence that any other authoritative body has demonstrated that rhLF is GRAS.

The data currently available in the published literature regarding the safety of rhLF are not adequate to meet the regulatory requirements for a GRAS determination. Although several companies are investigating rhLF, the existing published scientific evidence is not of the quantity and quality required by 21 C.F.R. 170.30(b) for a GRAS determination, even if corroborated by unpublished studies and other data and information. Nor can the safety of rhLF be demonstrated through experience when used in food. The recombinant product does not yet have a substantial

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[17] Medical foods are subject to special labeling requirements under 21 C.F.R. 101.9(j)(8), but are not exempt from the requirement that all ingredients be determined to be safe.

history of consumption in food by a significant number of consumers, as required by the regulations.[18]

There are little public data regarding the safety of consuming a larger quantity of human lactoferrin, whether natural or recombinant, than normally is present in the adult diet.[19] Although lactoferrin exists in breast milk, it is not a common component of the diet at any later stage in life. Milk products can contain small quantities of bovine lactoferrin, but this protein differs from human lactoferrin.[20] For instance, the amino acid sequences in bovine lactoferrin differ from the sequences in hLF,[21] and the products can have significantly different biological effects.[22]

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18 21 C.F.R. 170.30(c), (f).

19 On previous occasions, FDA has denied GRAS status for naturally-occurring products due to a lack of information on human use in the United States. For instance, FDA determined that inadequate data supported the safe use of miracle fruit and its extracts and concentrates. 39 Fed. Reg. 34468 (September 25, 1974); 42 Fed. Reg. 26467 (May 24, 1977).

20 FDA has expressed no objection to the marketing of lactoferrin derived from bovine milk (milk-derived LF) as an ingredient in "sports and functional foods." CFSAN Response Letter Re: GRAS Notice No. 000077 (August 14, 2001). FDA also expressed no objection to the marketing of an anti-microbial spray of milk-derived LF to be applied to beef carcasses that aLF Ventures self-determined as GRAS. The agency noted that the level of lactoferrin remaining on the beef was comparable to the amount naturally occurring in the beef. CFSAN Response Letter Re: GRAS Notice No. 000130 (August 21, 2003).

21 F.L. Schanbacher *et al.*, Bovine Mammary Lactoferrin: Implications from Messenger Ribonucleic Acid (mRNA) Sequence and Regulation Contrary to other Milk Proteins, 76 J. Dairy Sci. 3812 (1993); R.E. Goodman & F.L. Schanbacher, Bovine Lactoferrin mRNA: Sequence, Analysis, and Expression in the Mammary Gland, 180 Biochem. Biophys. Res. Commun. 75 (October 15, 1991).

22 For example, one researcher found that bovine lactoferrin helps halt the process of blood vessel development while human lactoferrin facilitates the growth of new blood vessels. K. Norrby, Human Apo-lactoferrin Enhances Angiogenesis Mediated by Vascular Endothelial Growth Factor A In Vivo, 41J Vasc Res. 293 (July - August 2004).

Accordingly, rhLF is not lawfully marketed as an ingredient in food until it is subjected to the premarket review and approval process for food additives under Section 409 of the FD&C Act. The product only can be marketed pursuant to a food additive regulation establishing the specific conditions under which the additive can be used in the food supply. Until that time, any food product containing rhLF as an additive is adulterated, in violation of Section 402(a)(2)(C) of the FD&C Act.

This result is consistent with the view expressed by FDA in a recent proposed rulemaking that a food additive regulation may be required for products created through recombinant technology:

> FDA recognizes that because breeders utilizing rDNA technology can introduce genetic material from a much wider range of sources than previously possible, there is a greater likelihood that the modified food will contain substances that are significantly different from, or are present in food at a significantly higher level than, counterpart substances historically consumed in food. In such circumstances, the new substances may not be GRAS and may require regulation as food additives.[23]

Finally, CFSAN must take into account the impact that any GRAS determination for rhLF would have upon the INDs for this substance. CFSAN therefore must coordinate with CDER on the consideration of any GRAS premarket notification of rhLF, in order to make certain that the safety requirements of the two Centers are consistent.

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[23] 66 Fed. Reg. 4706, 4711 (January 18, 2001)(internal citations omitted). Although some recombinant products have been determined to be GRAS, these products are distinguishable from rhLF in that the individual ingredients had long marketing histories demonstrating safety, and a substantial amount of data supported the safety of the recombinant form specifically. 55 Fed. Reg. 10932 (March 23, 1990); 58 Fed. Reg. 27197 (May 7, 1993)

IV. Dietary Supplement

Ventria also has suggested that it would promote rhLF for use in dietary supplements and that it currently is conducting clinical trials of this use. RhLF is excluded from the statutory definition of "dietary supplement," however, because it was not marketed prior to FDA acceptance of an IND to research the product as a drug.

The statutory definition of "dietary supplement" excludes articles:

> authorized for investigation as a new drug . . . for which substantial clinical investigations have been instituted and for which the existence of such investigations has been made public, which was not before such approval . . . or authorization marketed as a dietary supplement or as a food.[24]

Agennix has been conducting clinical trials on the use of rhLF as a drug for several years, and has made public these investigations. Results of a study conducted under an Agennix IND were published in 1999 in the journal Alimentary Pharmacology and Therapeutics,[25] and the company has published and presented other clinical trial findings on numerous subsequent occasions.[26] Two ongoing clinical trials sponsored by Agennix currently are listed on the NIH

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[24] Section 201(ff)(3)(A) of the FD&C Act; Pharmanex v. Shalala, 221 F.3d 1151, 1154 (10th Cir. 2000).

[25] A.R. Opekun *et al.*, Novel Therapies for Helicobacter Pylori Infection, 13 Aliment. Pharmacol. Ther. 35 (1999)(Attachment 5).

[26] The company has made presentations at, for instance, the Annual Meeting of the American Association for Cancer Research (AACR)(March 2004), the Wound Healing Society meeting (June 2003), and the American Society of Clinical Oncology (ASCO) meeting (2003), and has published results in the International Journal of Cancer, among others. A. Varadhachary *et al.*, Oral Lactoferrin Inhibits Growth of Established Tumors and Potentiates Conventional Chemotherapy, 111 Int'l J. Cancer 398 (2004).

clinicaltrials.gov website. Agennix also frequently issues press releases describing the company's activities, and stated as early as 2001 that:

> Agennix is a privately-owned biopharmaceutical company focused on research and development of recombinant human lactoferrin (rhLF) . . . and a variety of related peptides. . . . Agennix has completed numerous pre-clinical and clinical trials with rhLF demonstrating the enormous potential of lactoferrin in a wide range of clinical conditions.[27]

We are not aware of any evidence suggesting that human lactoferrin, whether from a natural source or derived through recombinant technology, was marketed as a dietary supplement or food prior to the initial IND submitted by Agennix in September 1996.[28] The cost of producing natural lactoferrin, at $3,600 per gram at 90 percent purity, has been prohibitive.

V. Conclusion

FDA should take appropriate action to ensure that Ventria and Pharming do not attempt to avoid the regulatory requirements applicable to rhLF for use in food or dietary supplements. The activities being conducted by Ventria and Pharming are cause for significant concern because the safety of their products has not been established. The testing or marketing of these products without appropriate oversight may put patients at risk.

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[27] Press Release, Agennix Receives Broad Patent Covering Production of Human Lactoferrin in Eukaryotic Cells (May 10, 2001)(Attachment 6).

[28] If rhLF had been marketed as a dietary supplement prior to the Agennix clinical trials, the product still could not be marketed without a 75-day premarket notification to FDA of intent to market a new dietary ingredient. We conclude that rhLF is a new dietary ingredient because we are not aware of any marketing of the ingredient prior to October 15, 1994. Nor is the ingredient exempt from the requirement on the basis of being extracted from human food.

As FDA has previously stated, allowing these articles to be marketed as food or dietary supplements would undermine FDA's regulation of new drugs. FDA has noted that Congress determined, in enacting section 201(ff)(3) of the FD&C Act, that allowing marketing of these types of products "would not be fair to the pharmaceutical company that brought, or intends to bring, the drug to market, and would serve as a disincentive to the often significant investment needed to gain FDA approval of new drugs."[29]

Sincerely yours,

Peter Barton Hutt
Ruth K. Miller

cc: Joseph R. Baca (HFS-600)
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[29] FDA, Final Decision in Pharmanex, Inc., Administrative Proceeding, Docket No. 97P-0441 at 4-5 (May 20, 1998).

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Sacramento Business Journal - April 9, 2004
http://sacramento.bizjournals.com/sacramento/stories/2004/04/05/daily44.html

SACRAMENTO Business Journal

LATEST NEWS

April 9, 2004

Regulators block plans for genetically altered rice

Celia Lamb
Staff writer

The U.S. Department of Agriculture has turned down a request by Ventria Bioscience of Sacramento to grow 120 acres of genetically altered rice in California for commercial use in pharmaceuticals, according to published reports.

Meanwhile, the state Department of Food and Agriculture has turned down a request from the company for "emergency" approval to grow its rice in California this year.

"What this means is we have a little more time now," said Ventria president and chief executive officer Scott Deeter of the state ruling. "We'll consider our options. We'll consider expansion in other areas outside California."

Deeter said the company received a letter from the USDA Tuesday asking the company to alter its rice-growing proposal by expanding the setback distance between Ventria's crop and other rice. Annual permits approved by the USDA since 1997 required a 100-foot setback for pharmaceutical rice grown for research, Deeter said.

Deeter said the company had changed and resubmitted its permit application to the USDA.

"This is part of a normal permitting process," he added.

Ventria's rice has been genetically altered to produce the human proteins lactoferrin and lysozyme, which have antimicrobial and anti-inflammatory properties. The Sacramento company has previously grown smaller plots of the rice in California for research and product development. Last year it had about 80 acres, Deeter said.

Ventria proposed expanding production by growing its rice in southern California counties that have no commercial rice production, a move intended in part to allay fears that the modified genes could come in contact with other rice crops.

Ventria plans to sell the rice to other companies for use in "medical foods," for iron-deficient women, children suffering from chronic diarrhea and patients with chronic diseases. The company hopes for US Food and Drug Administration approval within three years and eventual annual sales revenue of more than $500 million, Deeter said.

The California Rice Commission approved Ventria's plans last week and recommended emergency approval by the state, but some farmers worry the production of pharmaceutical rice could jeopardize the export market for their own crops.

In a letter to the Rice Commission, state Department of Food and Agriculture chief counsel John Dyer cited the

lack of federal permits as a reason for turning down the emergency request.

"The argued basis of the emergency is the loss of the current growing season," Dyer said in the letter. "However, it is unclear that anyone has received required federal permits, or is ready to plant the strain subject to these regulations, at anytime during the current growing season ... On the other hand, it is clear that the public wants an opportunity to comment prior to any authorization to plant."

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Sacramento Business Journal - April 19, 2004
http://sacramento.bizjournals.com/sacramento/stories/2004/04/19/story2.html

SACRAMENTO Business Journal

EXCLUSIVE REPORTS

From the April 16, 2004 print edition

Altered rice still headed to market

Rebuff may shift planting out of state

Celia Lamb
Staff Writer

Ventria Bioscience of Sacramento, turned down by regulators in its recent bid to start commercially producing its genetically altered rice in California, still plans to put its first product on the market in about two years.

Even if that means moving operations to another state or country.

"The time frame is such that we can step back and say, 'OK, let's look at what works for us,' " said Ventria president and chief executive officer Scott Deeter. "California's regulatory process is one of the challenges this state faces for biotechnology products."

Warnings of moves to friendlier terrain are common when businesses face off with regulators and legislators, but Ventria's plans for its modified rice -- designed to produce proteins for therapeutic use -- have been the subject of international scrutiny and organized opposition that raise the stakes for the local biotech startup.

One week ago the state Department of Food and Agriculture rejected Ventria's application to grow 120 acres of its biotech rice in California, double what it grew last year. The rejection sends the application back to a California Rice Commission advisory board for public review. Ventria also lacks a U.S. Department of Agriculture permit it would need to grow the crop.

Deeter said he expects the company will receive approvals from both the state and federal agencies in time to plant a crop this year, but if not, waiting until next year wouldn't delay product development. Ventria, he said, can scale up its rice production more than 50-fold in one growing season.

The company may also consider growing its rice outside of California. Deeter said possibilities include Texas, Arkansas, Missouri, Illinois, Florida or even South America, which has a winter growing season.

Great science, but maybe weak on PR: Ventria's rice contains a synthetic gene that lets the plants produce proteins found in human breast milk, said Ning Huang, the company's vice president of research and development. The company plans to extract the proteins, called lactoferrin and lysozyme, to use in nutritional supplements.

Initially the company plans to target a $1 billion market for iron supplements and a $500 million market for oral solutions to rehydrate people suffering from diarrhea, Deeter said. Eventually the company hopes to collaborate with companies that make nutritional drinks for children and the elderly. But first it needs approval from the U.S. Food and Drug Administration to sell the proteins for human consumption.

Ventria may need $10 million to $15 million in more funding to carry the company through commercialization of its first product, Deeter said, and then it would consider an initial public offering of stock.

"These guys have a nice product and a good market. I think they have a good chance of raising money," said Roger Wyse, a plant scientist and managing director of the San Francisco venture-capital firm Burrill & Co. "There's a fair bit of regulatory issues to go through, but two years is probably a reasonable time frame."

"They have great science, but I'm not sure they have done a very good job of managing public relations," Wyse added. "Earlier agricultural biotechnology products could have moved to market more quickly if they had paid more attention to public relations."

Growing concerns: Ventria, now with 19 employees, was founded in 1993 by University of California Davis molecular biology professor Raymond Rodriguez. He was chairman until 1999 and occasionally still advises the company on scientific matters.

Investors, mostly directors on the company's board, have put more than $20 million into Ventria, said Deeter, himself a board member. Other directors include Tom Urban -- former president, CEO and chairman of seed biotechnology company Pioneer Hi-Bred International Inc. -- and William Rutter and Pablo Valenzuela, co-founders of Chiron Corp. of Emeryville.

Ventria started growing small crops of genetically modified rice for research in 1997, creating consternation among biotech opponents and some rice farmers.

Opponents worry that Ventria's modified rice might show up in supplies of rice grown for food, either through interbreeding or mix-ups during handling.

They cite an October 2002 mishap in which some stalks of pharmaceutical corn were harvested along with a soybean crop in a Nebraska field. In that case ProdiGene Inc. of Texas had planted its corn the prior year for research, and remnants of the crop sprouted along with the soybeans planted in 2002.

ProdiGene had to buy 500,000 bushels of soybeans that might have come into contact with the corn.

Ventria has tried to address those concerns by proposing to grow the rice only in 10 California counties that have no commercial rice production, and by using equipment designated for use only with its own crop.

Cleared by a 6-to-5 vote: A state law passed in 2000 gives the California Rice Commission, an industry group funded by growers, the right to approve or reject protocols for growing more than 50 acres of biotechnology rice in California. The U.S. Department of Agriculture and the state Department of Food and Agriculture also must approve the plans.

If biotech rice mixes with rice for food production, it could severely damage California's export market to Japan, said Marysville rice farmer Charley Mathews, who sits on the Rice Commission's advisory board. But he voted to approve Ventria's proposal.

"The role of the advisory board is to set guidelines and protocols that assure us there's no possibility of contamination," he said. "We felt the probability of any of their crop ending up in commercial rice channels was about nil. We believe Ventria realizes the risks we have within the rice industry."

The commission's advisory board, however, was divided on the issue, approving the application on a 6-5 vote. The state's rejection last week means Ventria's proposal will have to go through a lengthier public review process that could take at least a month, Mathews said. Ventria would need approval within the next month or two to get a crop in the ground here this year.

By November, Ventria execs hope to pick a location for long-term rice production.

"We would like to have a location where we could scale up and we would invest a significant amount in infrastructure," Deeter said. That could include a processing plant and research operations, he added. The

company hopes to apply for USDA permits for that location by January, he said.

On Jan. 22, U.S. Agriculture Secretary Ann Veneman announced plans to update federal biotechnology regulations. Deeter said he expects that update would take effect by 2006.

"This company will be one of the first companies to have a food crop producing a therapeutic product under these new regulations," plant scientist Wyse said. "So that's a risk factor, obviously."

Adults, then infants: Ventria started selling rice-derived lactoferrin and lysozyme for research uses in the fourth quarter of 2003, Deeter said. The company took in more than $100,000 in revenue during first-quarter 2004, "but not a lot more," he added.

When derived from humans, the proteins can cost more than $30,000 per gram, but Ventria's proteins sell for less than 10 percent of that cost.

The company hasn't determined what it will charge for the proteins if they are approved for human consumption.

Only the seeds of Ventria's rice plants contain lactoferrin or lysozyme. Ventria harvests the rice, grinds it into flour and extracts the proteins. Ventria originally wanted to use the proteins in an infant formula but later changed its business plan to target adults and older children with nutritional deficiencies or disease.

"That's the proper way to go," said Bo Lonnerdal, a nutrition professor at UC Davis. "I believe these proteins are safe to include in infant formula, but this is a sensitive issue, so why not start with adults where the concerns are smaller?"

Ventria seeks a license from the FDA to sell its proteins as dietary supplements, said Delia Bethell, Ventria's vice president of clinical development. That's a faster process than obtaining approval for a new drug, but Ventria wouldn't be able to make claims about the health effects of its products.

The company has already begun clinical trials for a product containing purified lactoferrin saturated with iron. The hope is that the product will boost iron levels in women with mild deficiencies of the mineral. A Southern California doctor with a private practice is conducting the tests, Bethell said. Ventria has a confidentiality agreement with the doctor and would not disclose his name.

"About 19 percent of (U.S.) women are iron-deficient," Bethell said. "They don't always know it."

Human intestines have receptors for lactoferrin, Bethell added, and she believes the body could absorb the iron from lactoferrin better and with fewer gastrointestinal side-effects than the commonly used supplement ferrous sulfate.

A test in Peru: This summer Ventria plans to start testing a rehydrating drink in Peru on children with diarrhea. Deeter said Ventria is working with a company, which he would not name, that makes drinks from rice carbohydrates. "We believe we'll be using both lactoferrin and lysozyme in that product," Deeter said.

Lactoferrin suppresses intestinal inflammation, while lysozyme helps kill pathogenic bacteria. The company believes the proteins could offer long-term benefits for children afflicted with chronic diarrhea, which can damage their intestines and lead to malnutrition.

Malnutrition, in turn, makes the body more susceptible to the condition.

"It basically becomes a vicious cycle," Bethell said.

Over the next two years Ventria hopes to form a collaboration with a company that would incorporate lactoferrin and lysozyme in a nutritional drink for children or the elderly, possibly targeting the same market as existing drinks such as Abbott Laboratories' Pedialyte and Ensure, Deeter said.

The company would also likely license the use of its patents to other companies.

"The real issue here is for Ventria to get through the regulatory process," Deeter said. "These molecules are fairly well-known. There's more products to go after than we can handle."

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DUPLICATE

Pharming Announces Positive Results Of Study With Human Lactoferrin

Leiden, November 24, 2004. Pharming Group N.V. ("Pharming" or "the Company") (Euronext: PHARM) announced today the positive results from a key study with recombinant human lactoferrin (rhLF). The Company will use these results for Generally Regarded as Safe (GRAS) registration of rhLF for nutritional applications.

The results demonstrate that rhLF can be consumed orally at high amounts with no adverse effect. Pharming has conducted the extensive animal toxicology study in cooperation with the TNO Institute for Nutrition and Food Research to observe the effect of the oral intake of rhLF. After publication of the study results, the Company will prepare its GRAS filing along with an expert opinion on use of rhLF for nutritional applications.

"I am very pleased with the positive outcome of this study with recombinant human lactoferrin, as well as the positive results in animal studies with human fibrinogen," said Dr. Francis J. Pinto, CEO of Pharming. "Based on these achievements, Pharming will consider making additional investments to accelerate the development of these innovative products."

Recently, Pharming completed initial studies in animal models with recombinant tissue sealant / fibrinogen (rhTS / rhFIB). The results of these studies indicate that rhTS / rhFIB is effective in stopping bleedings and may provide advantages over commercially available plasma fibrin sealants. The Company has started licensing discussions with several parties to accelerate commercial production of these products.

Recombinant Human Lactoferrin

Human lactoferrin is a natural protein that helps to fight and prevent infections and strengthens the defense system of the human body. The protein is present in substantial quantities in mother's milk and plays an important role in the defense system of infants. The protein is also present in various body fluids and continues to play an important role against a wide range of bacterial, fungal and viral pathogens in adults.

Pharming is developing recombinant human lactoferrin (rhLF) as a nutraceutical and intermediate while evaluating applications of the product for the pharmaceutical market. Pharming has demonstrated that rhLF is safe, effective and comparable to the natural hLF. Pharming plans to file for Generally Regarded as Safe (GRAS) status for rhLF and commercialize the product for nutritional applications.

Background on Pharming Group N.V.

Pharming Group N.V. is developing innovative protein therapeutics for unmet medical needs. The Company's products include potential treatments for genetic disorders and specialty products for surgical indications. Pharming's lead product for hereditary angioedema is in Phase III of clinical development. The advanced technologies of the Company include novel platforms for the production of protein therapeutics, as well as technology and processes for the purification and formulation of these products. Additional information is available on the Pharming website, http://www.pharming.com

This press release contains forward looking statements that involve known and unknown risks, uncertainties and other factors, which may cause the actual results, performance or achievements of the Company to be materially different from the results, performance or achievements expressed or implied by these forward looking statements.

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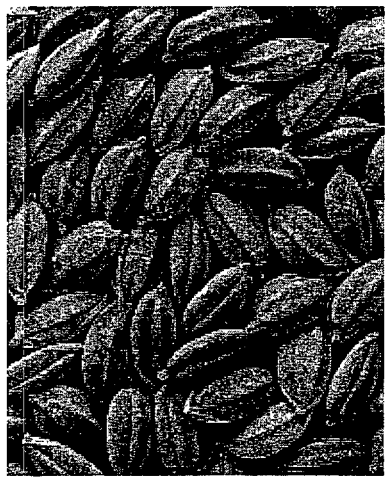
High-Level Protein Expression System Uses Self-Pollinating Crops As Hosts

Ning Huang

Bacterial, fungal, and cultured cell expression systems have been used to produce a variety of recombinant proteins, and they play an extremely important role in biopharmaceutical production. However, because of limitations of cost and scale, all those systems are facing challenges in meeting the high-volume demand of some proteins. Amgen's drug Enbrel (www.enbrel.com) is one recent example. Plants, with their potential to produce large volumes of recombinant proteins at low cost, are considered by some to be an excellent alternative to the common expression systems used to produce recombinant proteins (1).

Since 1997, Ventria Bioscience (www.ventria.com) has worked to develop its version of the concept, leading to the ExpressTec high-level protein expression system, which uses the self-pollinating crops of rice and barley. We have achieved recombinant protein expression at up to 1% of the rice grain's weight. For many candidate proteins, the expression level is between 0.1 and 1.0% of grain weight. Such high expression levels, coupled with the large-scale production of cereal grains, could make the transgenic production of large volumes of recombinant proteins and peptides a reality. Our system offers several advantages: high and stable expression levels of recombinant proteins, tissue-specific expression in the grain endosperm, rapid scalability to metric-ton quantities, prevention of gene flow with self-pollinating crops, low capital investment and production costs, and efficient processing and recovery.

Rice Life Cycle Suits Recombinant Protein Production: The rice plant's life cycle begins with seed germination, followed by a vegetative stage, a reproductive stage, and a ripening stage. The vegetative stage is about 60–100 days, during which the rice plant develops and generates tillers, stalks that sprout up from the base of the plant. A single rice plant could have as many as 100 tillers producing more than 10,000 rice grains, which is important for early generation seed increases and producing small quantities of recombinant protein rapidly for feasibility studies.



Transgenic rice grain expressing recombinant human lactoferrin

When the rice plant enters its reproductive stage, the panicle (a loosely branched flower cluster) grows. Over 99% of cultivated rice flowers are self-pollinated because of the floral architecture, which is not receptive to pollen from other plants. Also, rice pollen grains are relatively short-lived, losing their viability within five minutes of shedding from the anther, which leads to an extremely low rate of cross-pollination. No outcrossing rates beyond 10 meters have been observed. Thus the isolation distance for transgenic rice that would be required by regulatory agencies is significantly lower than that for cross-pollinating plants such as corn and tobacco.

PRODUCT FOCUS: RECOMBINANT PROTEINS

PROCESS FOCUS: PROTEIN PRODUCTION AND PROCESSING

WHO SHOULD READ: PROJECT MANAGERS; PROCESS AND PRODUCT DEVELOPMENT; BUSINESS DEVELOPMENT FROM ENGINEERS AND SCIENTISTS TO CHIEF TECHNICAL OFFICER

KEYWORDS: PROTEIN EXPRESSION, CEREAL GRAIN, BIOPROCESSING, INDUSTRIAL BIOTECH, NUTRACEUTICALS, ENZYMES, TRANSGENIC PLANTS

LEVEL: INTERMEDIATE

Rice grains ripen after pollination. The ripening stage takes 30–50 days. This is when starch and storage proteins accumulate, and the grains are desiccated after filling out. Recombinant proteins expressed in rice accumulate in the grains at this time as well. Rice is then harvested and stored after being dried to a moisture content of 14% or less. The grain can be stored for three to five years without losing viability if it is kept below 13.5% moisture content. Recombinant proteins in the rice grains are also stable in excess of three years.

Storage Protein Gene Expression Is the Foundation: A rice grain consists of an inedible outer husk (20% of grain weight) and the enclosed brown rice (80%), which in turn contains an embryo (2%), an aleurone layer (7%), and endosperm (91%). Aleurone is outer-layer protein-rich cells. The major component of brown rice is starch. Almost all of its 8% protein content comes in the form of four major types of storage proteins: glutelin (>80%), globulin (10%), prolamin (<5%), and albumin (5%). Stored in type I and type II protein bodies, rice proteins are usually encoded by large gene families. Both glutelin and globulin deposit into type II bodies. An estimated 20 genes code for glutelin in rice. On the other hand, the plant has only a single gene for globulin.

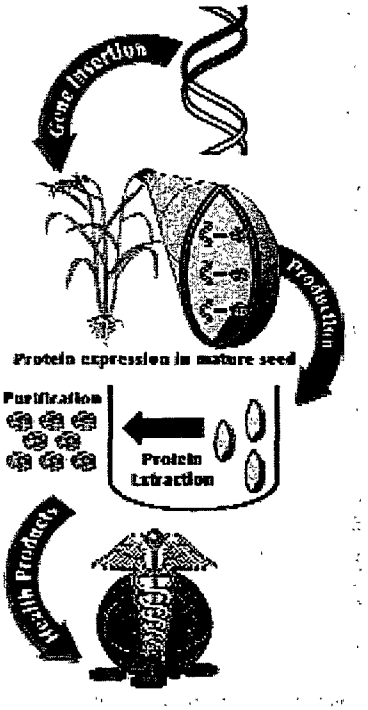
Rice storage protein genes begin to express five days after fertilization, peaking at 20–30 days depending on the environment and the variety of rice. Messenger RNA transcribed from the storage protein genes is differentially distributed to subdomains of the endoplasmic reticulum (ER). The proteins are synthesized and guided by a signal sequence into the ER through a translation/translocation process. During that process, the signal sequence is cleaved off to leave an intact mature protein. Storage proteins such as prolamin are retained inside the ER to later form a type I protein body within its membrane through budding. Glutelin and globulin deposit into a type II protein body, a single-membrane–bound organelle.

Evidence of high-level expression and accumulation of rice storage proteins in the grains led us to believe that recombinant protein could be expressed at similarly high levels using promoters and signal sequences from these storage proteins.

Glycosylation Pathway and Glycan Structure: In the ER, protein is attached with a 10-mannose chain through N-linked glycosylation at the amide residue of asparagine. Some mannose residues are removed from the chain later in the protein sorting pathway, where new sugars such as xylose, fucose, and glucose are added. A typical plant glycan carries one fucose and one xylose molecule. Recombinant proteins expressed in rice grains would bear the typical plant glycan structure. This has been confirmed by the glycan structure determination of human lactoferrin expressed in rice grains. Three major types of glycans are present in recombinant human lactoferrin, and all of them carry the typical core structure of the plant glycan with both xylose and fucose. None carries sialic acid as in the native form of human lactoferrin.

There has been recent attention to the importance of correct glycan structures on human therapeutic proteins. Clearly, certain protein targets require human glycans for optimal efficacy and stability when reintroduced into the human system. Alternatively, many other molecules may tolerate plant glycans with no effect on function. Regarding plant glycan immunogenicity issues, humans are exposed to plant glycan structures throughout their lives, especially through food ingestion. There should generally be little effect of plant glycan structures in plant produced proteins, espcially in oral and topical therapeutic and nutritional applications.

Figure 1: Schematic diagram of ExpressTec



The **ISOLATION** distance for transgenic rice that would be required by regulatory agencies is significantly lower than that for cross-pollinating plants such as corn and tobacco.

EXPRESSION

Strong promoters are needed for high-level expression of recombinant proteins, so we tested various rice storage protein promoters along with a signal sequence. Genes of interest were placed downstream of the signal sequence, and the final product was expressed as a fusion protein. To select for strength, we tested six promoters isolated from rice storage

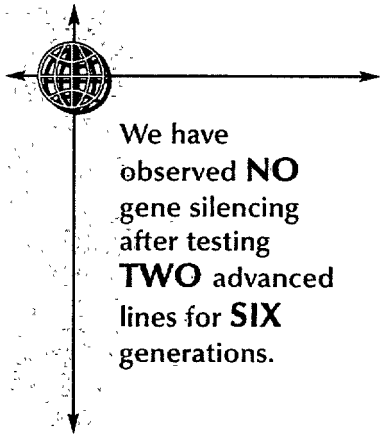
protein genes. All six constructs were identical except for the promoter sequences. The same signal sequence was included in each construct because protein stability would be lower in the cytosol (cytoplasmic fluid) than if the protein were deposited in an organelle such as the protein body. We used the gene for human lysozyme as our target gene.

Transgenic grains from each line were analyzed for the expression of recombinant human lysozyme. The glutelin 1 (Gt1) promoter caused higher expression levels of human lysozyme than the other five promoters, ranging from 4.5% to 40% of total soluble protein with an average of 14%. Expression levels from the globulin (Glb) promoter varied from 3% to 23% with an average of 13%, which was very close to that achieved with the Gt1 promoter. Three other promoters derived from rice glutelin genes were able to direct human lysozyme expression to about 10% of total soluble protein, all lower than that controlled by either Gt1 or Glb promoter.

Next, we compared the strength of the Gt1 and Glb promoters in expressing various genes and found that Gt1 was stronger. Thus we consider the Gt1 promoter to be the strongest promoter for recombinant protein expression in rice. Our expression system uses such a strong promoter along with a signal sequence, a codon-optimized gene of interest, and sometimes (if the gene of interest codes for a small peptide) an additional fusion partner.

One way to illustrate our level of recombinant protein expression is to compare the expression of the same protein by various plant species. Table 1 shows the expression of human lactoferrin in five plant hosts. Human lactoferrin is expressed in our rice grain at a level 10 times higher than in rice grain using other systems — and at least 30 times higher than in tobacco. Another protein we have tested is human lysozyme. The expression level of human lysozyme using our system has reached 1% of the rice grain weight or 40% of the total soluble protein, which is 400 times higher than that expressed in the tobacco plant (2). California rice yields are 7200–8000 kg of brown rice per hectare of cropland. With our expression system, that would suggest a potential yield of human lysozyme at 72–80 kg/ha.

Our expression of recombinant proteins is also highly tissue-specific. Recombinant protein expression under control of the Gt1 promoter is present only in the rice grain and not detectable in any other plant tissues such as the root, stem, young leaf, mature leaf, or anther.



We believe that the high level of protein expression possible with the ExpressTec system can be attributed to its capability for targeting recombinant protein into the protein bodies (organelles) where it is sequestered and protected from protease digestion. The signal sequence plays an important role in directing recombinant proteins into the ER and then through the various protein sorting systems within the rice cells to reach the protein body. Electron-microscopic evidence shows that rice endosperm possesses a highly adaptive capability to store recombinant proteins. A comparison study showed that the expression level of recombinant protein in barley grains with a signal sequence is at least 10 times higher than without the signal sequence (3). Evidently, recombinant proteins in cytosal are much more susceptible to protease digestion.

Production of Metabolites: Our system is not used only to produce recombinant proteins of pharmaceutical and nutraceutical importance, but also to produce secondary metabolites through metabolic engineering. One such product is matairesinol, a type of plant lignan (which are a group of phenypropanoid natural products, usually dimers or oligomers). Consumption of matairesinol is associated with reduced risk of breast cancer, prostate cancer, and colon cancer. To produce the substance in rice, four genes catalyzing the matairesinol synthesis pathway were expressed. Analysis indicated a significant elevation of matairesinol in the rice grains.

Recombinant Proteins Expressed in Rice Grains Are As Active As Native Proteins: We have produced human lactoferrin, human lysozyme, and many other proteins in rice grain (Figure 1). After purification, the biophysical and biochemical characteristics of those recombinant proteins were compared to those proteins derived from native sources. We have found that recombinant proteins and their native counterparts have the same molecular weight, N-terminal sequence, enzymatic activity, surface charge (an indication of same three-dimensional structure), and surface structure as indicated by reaction to specific antibodies. In addition, we found that they possess the same antibacterial activity, pH stability, and resistance level to protease activity. We have been unable to find any difference between recombinant proteins expressed in rice grains and their native counterpart except for the different glycosylation pattern.

Stable Recombinant Protein Expression Over Generations: High-level expression of recombinant proteins in plant cells may lead to gene silencing, so expression stability of recombinant proteins over generations is a concern. Our

Table 1: Expression of human lactoferrin in various plant species (TSP = total soluble protein)

Crop	Tissue	Expression Level (% TSP)	Expression Level (% Biomass)	Reference
Tomato	Fruit, Leaf	Detectable	Detectable	4
Tobacco	Culture cell	Detectable	Detectable	9
Tobacco	Leaf	0.8	Detectable	6
Tobacco	Leaf	0.3	Detectable	7
Corn	Grain	Detectable	Detectable	8
Potato	Tuber, Leaf	0.1	Detectable	9
Rice	Grain	Detectable	0.05	4
Rice (ExpressTec)	Grain	25	0.5	10

system seems to represent an exception to the rule. We have observed no gene silencing for the expression of human lactoferrin and lysozyme after testing two advanced lines (one expressing human lactoferrin and one expressing human lysozyme) for six generations. Expression of human lactoferrin remains at 0.5% of brown rice weight, and the expression of human lysozyme is at 0.6% of brown rice weight.

Why do we see such stable expression? Our hypothesis is that rice endosperm cells play the role of nutrient storage for seed germination and thus enter into a self-destruction stage (apoptosis) after starch and protein have accumulated. High-level expression of recombinant proteins (and their accumulation in the protein bodies) would not impose any physiological pressure to the cells. Thus, the rice plant does not need to activate a cell defense system such as methylation. On the other hand, the expression level will have a limit beyond which it could negatively affect seed development and germination. We have not yet discovered this limit but assume that it is somewhere between 1.0 and 1.5% of brown rice weight, depending on the protein expressed.

Storage and Processing

One advantage of using rice as the host for recombinant protein expression is that rice proteins are stable in stored grain. As mentioned above, rice grain can be stored for three to five years without losing viability if stored under ideal temperature and moisture conditions. We have tested the stability of human lysozyme and lactoferrin in rice grains stored for over 2.5 years and found no protein loss. Because of this high degree of stability, large amounts of rice can be stored to provide a continuous supply of grain for processing and manufacturing of pharmaceutical and nutraceutical products.

With human lactoferrin, simple extraction of brown rice flour recovers most human lactoferrin from the grain at a concentration of about 25% of total soluble protein. Filtered extract can be loaded directly onto a Sepharose column from Amersham Biosciences (www.amershambiosciences.com). Human lactoferrin eluted from that single column is greater than 90% pure, with a high recovery rate (>50%) of the final product in lyophilized powder.

Similarly, our lines of transgenic rice express human lysozyme as high as 50% of the total soluble protein, providing for a simple and efficient recovery process that can be scaled up easily. To extract recombinant proteins from rice grains, the rice is ground down to 20–100 mesh size, then the protein is extracted with a buffer containing salt. Most recombinant proteins are water soluble, so organic solvents and detergent extraction are not needed. Rice proteins are less soluble at low pH values. Therefore, if the recombinant protein can survive low pH conditions, it can be enriched by a low pH extraction step.

Economics

Rice production costs and yields vary from ecosystem to ecosystem and from country to country. Our cost estimates (in US dollars) for rice production are based on the world price of commodity rice, about $120/1000 kg of paddy rice. Of course, growing bioengineered rice to produce pharmaceutical and nutraceutical proteins would require higher capital investment than producing commodity rice. The higher input in producing plant-made pharmaceuticals includes more intensive care of the plant, segregation from other rice, dedicated equipment for planting, harvesting, drying, storage, and milling — as well as documentation and stringent regulatory compliance. We estimate the cost of rice production for pharmaceuticals at about three times the commodity rice production: $360/1000 kg paddy rice. With our system, the expression level of recombinant protein is about 0.5% of brown rice weight, which translates to $90/kg of recombinant protein from the rice grain.

In California, commodity rice production is on the high end of costs ($2000/ha or $800/acre), but the average yield (9659 kg/ha or 8500 lbs/acre) is also very high, resulting in reasonable rice production costs per kilogram ($207/1000 kg paddy rice). Increasing threefold that cost for production of rice-made pharmaceuticals would put it at about $621/1000 kg or about $155/kg of recombinant protein if expressed at 0.5%.

Cost of Processing: Cost associated with processing rice grain will largely depend on the final product and whether it is an injectible, oral, or skin-care therapeutic product, a nutraceutical, or an industrial purpose. It will cost much less to make a functional food than to prepare a high-purity pharmaceutical. To prepare an extract from rice flour expressing

MARTIN DEWIT (WWW.ISTOCKPHOTO.COM)

lactoferrin for functional food use, food industrial processing would be used at a cost of about $0.50 to 1.00/kg flour. Higher purity products are also very efficiently produced using a single column process. Greater than 90% pure human lactoferrin is produced and the process can be easily scaled up to meet increaing demand. We estimate that it would cost about $5 to $10 to generate one gram of human lactoferrin from rice flour ina cGMP facility operating at a scale of 600 kg/year.

By-Product Use: During the processing of rice flour, 98% of the biomass remains as a by-product. This cake is rich in starch and protein thus could provide an alternative biomass source for ethanol production. This revenue potential is not considered in the above economics.

CONSIDER THIS . . .

By exploiting the nature of cereal grains, we have developed the ExpressTec recombinant protein expression system for producing pharmaceutical, nutraceutical, and industrial enzymes. Our system could provide several advantages over others in expression level, scalability, cost of production, and processing ease. In addition, the system is more environmentally sound than many others because it uses a self-pollinating crop. Data show expression levels of recombinant protein as high as 1% of rice grain weight, which allows it to be easily processed and recovered. Considering all of the above, we believe ExpressTec deserves consideration for production of recombinant proteins and peptides requiring large volumes and low cost of production.

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DUPLICATE

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Novel therapies for Helicobacter pylori *infection*

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SUMMARY

Background: Increasing antibiotic resistance has begun to impair our ability to cure *Helicobacter pylori* infection.
Aim: To evaluate orally administered novel therapies for the treatment of *H. pylori* infection.
Methods: Healthy *H. pylori* infected volunteers received: (a) hyperimmune bovine colostral immune globulins, (b) an oligosaccharide containing an *H. pylori* adhesion target, Neu5Aca2-3Galb1–4Glc-(3′-sialyllactose), or (c) recombinant human lactoferrin. Outcome was assessed by urea breath test or histological assessment of the number of *H. pylori* present.
Results: None of the novel therapies appeared effective and no adverse events occurred.
Conclusion: Although *in vitro* data appeared promising, *in vivo* results were disappointing. Higher doses, longer duration of therapy, adjunctive acid suppression, or a combination could possibly yield better results.

INTRODUCTION

Helicobacter pylori occupies a niche where it is largely protected from competition by micro-organisms and from the host immune response.[1] *H. pylori* infection is associated with a brisk acute and chronic inflammatory response.[2, 3] Effective treatment of the infection has proven difficult, in part because of the different micro-environments where *H. pylori* may live (e.g. free in mucus, attached to epithelial cells, in different pH, etc.).[4] Current therapies report variable cure rates.[5] The reasons why therapy fails remain unclear, but bacterial resistance and poor compliance to the often complicated drug regimens have proven important.[6]

The ideal therapy would cure the infection without the development of antibiotic resistance in *H. pylori* or other flora. Therapy that disrupts the ability of *H. pylori* to colonize the gastric mucosa, impair its living conditions, limit its ability to garner essential nutrients, or minimize its ability to evade the immune response, may have great therapeutic potential. Therapies that disrupt the local haemostasis in chronic *H. pylori* infection should also provide insight into the pathogenesis of *H. pylori* disease.

The *H. pylori* organism has a number of virulence factors important for colonization and establishing the chronic infection of the stomach. Putative virulence factors include urease, proteases, phospholipases, flagella and various adhesins.[7–12] Urease is thought to help in maintaining the pH of the mucosal micro-environment to within a suitable range.[13] Urease may provide an antigen for antibodies to bind and help in eliminating the infection. In additon, urease can be inhibited by anti-urease immunoglobulins, predominantly by steric hindrance at antigenic sites other than the catalytic site.[14] The adherence of *H. pylori* to the gastric epithelium is important for its colonization. Bitzan *et al.* have recently demonstrated that bovine colostrum can inhibit the binding of *H. pylori* as well as

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surface epithelium receptors phosphatidylethalnolamine, gangliotetraosylceramide, and gangliotriaosylceramide.[15]

We evaluated the role of three novel orally administered therapies: (a) various anti-*H. pylori* immune globulins, (b) an oligosaccharide containing the active site of an adhesin, Neu5Aca2-3Galb1-4Glc-3′-(3′-sialyllactose), and (c) recombinant human lactoferrin (rhLf).

In a manner similar to the treatment of other enteric pathogens,[16] orally administered bovine immunoglobulins specifically designed to interact with various *H. pylori* epitopes were tested in infected human subjects.

H. pylori contains several adhesins,[17–21] including a sialic acid binding adhesin for which the oligosaccharide 3′-sialyllactose (NeuAca2-3Galb1-4Glc) has been identified as the ligand.[15] 3′-sialyllactose occurs naturally in human milk[22] and has been shown *in vitro* to prevent the initial attachment of *H. pylori* to monolayers and to detach a significant number of *H. pylori* organisms from HuTu-80 monolayers.[23] It was hypothesized that oral administration of 3′-sialyllactose would reduce the number of *H. pylori* organisms attached to human gastric mucosa *in vivo*.

Lactoferrin (Lf) is a glycoprotein found in high concentrations in human milk. It is also found, at lesser concentrations, in exocrine secretions and in polymorphonuclear leucocytes.[24] Lf has potent antimicrobial properties *in vitro* and *in vivo*, and several mechanisms appear to contribute to its antimicrobial function.[25, 26] A bacteriostatic function is provided by the two iron-binding domains of the Lf molecule, and this theoretically deprives the bacteria of nutrient iron that is necessary for growth.[27] A direct bactericidal effect for *H. pylori* has also been described.[28, 29] The N-terminus of the protein molecule appears to cause harmful changes to bacterial membrane permeability and the release of lipopolysaccharide from the outer membrane.[30] *In vitro*, Lf has demonstrated antibacterial activity against enterotoxigenic *Escherichia coli* 0111 and *Shigella flexner*, small bowel and large bowel pathogens, respectively.[31]

Recently, pure recombinant human lactoferrin (rhLf), produced synthetically in reactors by *Aspergillus awamori*, has become available in large quantities.[32, 33] It was hypothesized that if intraluminal iron could be bound and/or the bacterial membrane permeability could be altered by an oral dose of rhLf, the augmentation of host neutrophil stimulation could occur, and the *H. pylori* infection might be cured or suppressed.

METHODS

Orally administered bovine anti-H. pylori immunoglobulin

Three anti-*H. pylori* iso-types were produced by hyperimmunized dairy cows. The cows were previously hyperimmunized with target antigens during pregnancy. Collectively, these products are refered to as specific bovine immune concentrate (BIC) (Galagen Inc., Arden Hills, MN). The first experiment was performed as three open-label studies designed to assess the safety and efficacy of a therapeutic regimen of oral administration of three BIC preparations containing specific IgG immunoglobulins. The BIC products were prepared from the first six post-partum milkings using as antigens: (i) jackbean urease (aJBU), (ii) a glycerolipid receptor-specific *H. pylori* adhesin (aHpA), and (iii) a formalin-killed whole *H. pylori* bacteria (aWCP). The IgG antitarget potencies were 1:6400 against jackbean urease, 1:6400 against *H. pylori* HpA adhesin, and 1:3200 against whole-bacterial-cell *H. pylori*. Jackbean urease shares a 57% primary amino acid sequence homology with *H. pylori* urease.[34] Bovine anti-jackbean urease immunoglobulin binds to *H. pylori* urease, as has been measured by enzyme-linked immunoassay, and also partially inhibits ammonia formation when measured in a colorimetric assay. Bovine immunoglobulins directed against formalin-killed *H. pylori* demonstrate a dose-dependent killing of *H. pylori* as measured by a decrease in colony forming units (unpublished data).

H. pylori infected, but otherwise healthy, subjects were recruited and were admitted to the research unit. Active *H. pylori* infection was defined as typical histopathology, positive serology (HM-CAP, Enteric Products Inc., Westbury, NY) and a positive ^{13}C-urea breath test (Meretek Diagnostics, Houston, TX). The outcome variables were cure or suppresion of infection, safety and tolerability. Exclusion criteria included a history of milk intolerance, the use of antimicrobial or bismuth salt agents within 3 months of entry into the study or during the study periods, ongoing use of nonsteroidal anti-inflammatory agents, corticosteroids or ethanol. No subject consumed antacids during the study periods.

Subjects received three standardized meals per day. Three subjects received 7.8 g antiurease BIC administered 1 and 3 h after meals and just before bedtime for

2 days (14 doses). Six subjects received 7.6 g antiadhesin BIC, also administered 1 and 3 h after meals and just before bedtime for 2 days (14 doses). Six subjects received 3.7 g anti-whole cell BIC administered 1 h after meals and just before bedtime for 2 days (eight doses). BIC was suspended in 240 mL of water flavored with commercial fruit punch mix (Kool-Aid, Kraft General Foods, White Plains, NY) and mixed at the bedside with an electric mixer. The BIC was consumed within 2 min of mixing.

Subjects were monitored for adverse events during and after BIC administration. Total serum antibovine IgG antibody titres and plasma levels of the three BIC immunoglobulins were measured before and after BIC administration (Scicor Inc., Indiannapolis, IN).

^{13}C-urea breath testing was performed on the morning following the last dosage of BIC (day 3), along with routine post-study safety evaluations. If the ^{13}C-urea breath test was negative post-therapy it was repeated on day nine to test whether infection had been cured, rather than suppression.[35]

Orally administered H. pylori *adhesin target*

An open-label study was performed to assess whether the oral administration of the oligosaccharide, 3′-sialyllactose (Neose Technologies Inc., Horsham PA) would decrease the number of *H. pylori* organisms attached to human gastric mucosa.

Healthy adult male subjects with active *H. pylori* infection (positive *H. pylori* serology and ^{13}C-urea breath test) underwent upper gastrointestinal endoscopy, with large-cup gastric mucosal biopsies taken from specified locations. The biopsies (two from each site) included: the greater curve antrum within 2 cm of pylorus (site A4), angulus (site A3), and greater curvature of the mid corpus (site B6). The density of the attached bacteria was quantified histologically using Genta stain and recorded as the number of organisms per high power microscopic field using the scale of 0 (none) to 5 (numerous), as previously described.[36]

Two weeks after the biopsy sites had healed, the subjects were admitted to the research unit. 3′-sialyllactose, 2 g dissolved in 15 mL distilled water, was administered by mouth for five doses immediately after a standardized meal or snack (at 08.30 hours, 12.20, 18.30, at 21.50, and 1 h before endoscopy on the following morning). A total of 10 g 3′-sialyllactose was administered to each subject. A second endoscopy with biopsies was performed to ascertain whether there was a change in the number of attached *H. pylori* or in the inflammatory response. Identical biopsy processing and analysis procedures were used.

Early studies with 3′-sialyllactose suggested that serum liver transaminase may be mildly elevated after treatment. Blood samples were obtained from each subject for liver transaminase testing before receiving the 3′-sialyllactose and again at the time of the second endoscopy.

Oral administration of recombinant lactoferrin

Two open-label studies (low and high dose) were performed to assess the safety and efficacy of orally administered rhLf (Agennix Inc., Houston, TX) to cure or suppress *H. pylori* infection in adult subjects. *H. pylori* infection was ascertained by positive *H. pylori* serology and ^{13}C-urea breath test. The outcome variables tested included efficacy in treating *H. pylori* gastritis, safety, serum ferritin levels and plasma iron levels before, during and after receiving rhLf.

Each subject was admitted to the clinical research facility by 07.30 hours on the first study day after an overnight fast. The rhLf was administered by mouth five times throughout a 24-h period. Each dose of rhLf was mixed with 60 mL of whole milk until the rhLf was completely suspended (about 1 min). The suspension was consumed within 2 min of preparation. The first dose was given at breakfast time (t = 0 h) followed by additional dosing at t = 4 h (lunch-time), t = 9 h (supper-time), t = 14 h (bedtime), and t = 24 h (breakfast time on day 2). Six subjects received 250 mg per dose and six subjects 1 g per dose.

A ^{13}C-urea breath test was administered 2 h after the second dose (t = 6 h) and repeated 4 h after the fifth dose (t = 28 h). Standardized meals and snacks were provided, beginning 2 h after the inital rhLf administration to approximate a total caloric intake of 2400 kcal/day. A positive result was defined as a change in the urea breath test from positive to negative (enrichment change 2.4‰).

All studies were approved by the Baylor Affiliates Review Board for Human Studies and all subjects granted informed written consent prior to participation in the respective studies.

Statistical analysis

The sample sizes were based on the availability of the test compounds and the 95% confidence intervals. The upper 95% C.I. for the smallest group ($n = 6$) for zero successes from six attempts is 45%. The upper 95% C.I. of the larger samples is progressively smaller. Statistical analyses were done using SIGMASTAT 2.03 (SPSS Inc., Chicago IL).

RESULTS

*Bovine anti-*H. pylori *IgG*

Fifteen individuals (eight men, seven women; ages 24–67 years) received bovine immune concentrate. No subject, in any test group, cleared their *H. pylori* infection as indicated by ^{13}C-urea breath test after 3 days of therapy (Figure 1). Quantitative serum antibovine immunoglobulins were measured pre- and post-dosing and the results were unchanged, mean 1539 and 1642 g/mL for pre-BIC administration, and post-BIC administration, respectively ($P = 0.44$). No increase in plasma levels of the three bovine immunoglobulins (aJBU, aHpA, aWCP) were detected (Table 1).

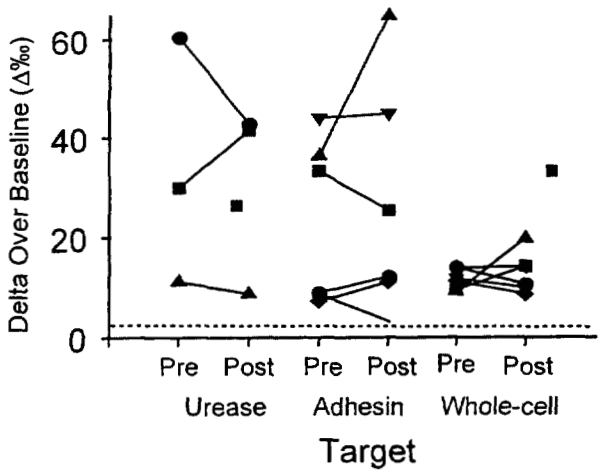

Figure 1. The results of the urea breath testing before and after therapy with oral bovine immune concentrate to jack bean urease, anti-*H. pylori* adhesin, or whole cell preparations. In no instance did the test become negative nor were consistent decreases in urease activity seen. The dotted line represents the cut-off value between a postive and a negative test.

Table 1. Serum bovine immunoglobulin levels before and after oral administration to volunteers

BIC subtype and subject number	Pre-therapy quantitative antibovine-IgG immunoglobulin (mg/dL)	Post-therapy quantitative antibovine-IgG immunoglobulin (mg/dL)
aJBU-BIC 1	1595	1113
aJBU-BIC 2	871	695
aJBU BIC 3	1722	1575
MEAN aJBU	1396 + 254	1128 + 265, $P = 0.13$
aHpA-BIC 4	1845	1303
aHpA-BIC 5	1074	818
aHpA-BIC 6	5270	5910
aHpA-BIC 7	1360	1194
aHpA-BIC 8	696	887
aHpA-BIC 9	627	523
MEAN aHpA	1812 + 715	1772 + 835, $P = 0.82$
aWCP-BIC 10	502	505
aWCP-BIC 11	1608	1808
aWCP-BIC 12	973	872
aWCP-BIC 13	539	614
aWCP-BIC 14	1443	1297
aWCP-BIC 15	2685	5525
MEAN aWCP	1292 + 334	1770 + 776, $P = 0.36$

3′-sialyllactose administration

Six men (age 33–49 years) received oral 3′-sialyllactose. There were no adverse reactions. There were no changes in *H. pylori* organism adherence (counts per field) to the gastric mucosa (Figure 2) or in the intensity of the inflammatory response for either acute or chronic inflammatory cells (data not shown). There were no significant changes in serum liver transaminase tests after 3′-sialyllactose therapy (data not shown).

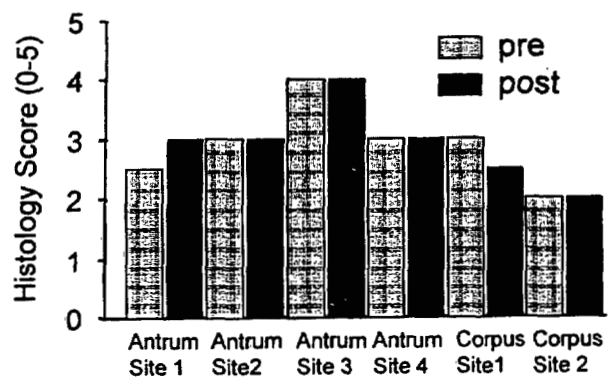

Figure 2. Median score (0–5) for *H. pylori* organisms by histology before and after oral therapy with 3′-sialyllactose (P = n.s.)

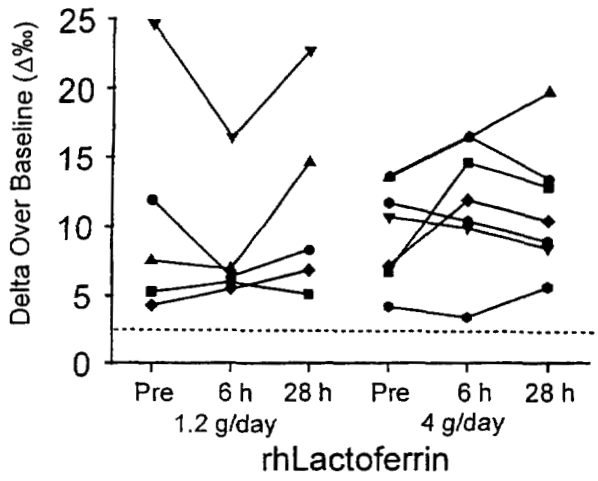

Figure 3. The results of the urea breath testing before and after therapy with either 1.25 or 5 g of orally administered recombinant human lactoferrin. No change in urease activity was seen. The dotted line represents the cut-off value between a postive and a negative test.

Recombinant lactoferrin

Twelve individuals (nine men, three women; ages 22–63 years) received rhLF. No subject cleared their infection, as indicated by persistent positive ^{13}C-urea breath test results (Figure 3). No significant adverse events were observed and no change occurred in serum ferritin, mean = 102.7 ng/mL ± 28 (± S.E.M.) before therapy compared with mean = 96.5 ng/mL ± 25 (± S.E.M.) after initiation of therapy ($P = 0.59$) or serum iron, mean = 102.4 μg/dL ± 19 (± S.E.M.) before therapy compared with 112.1 μg/dL ± 24.5 (± S.E.M.) after initiation of therapy ($P = 0.74$).

DISCUSSION

These studies demonstrate the difficulties that are encountered in treating *H. pylori* infection. Under the conditions tested, none of the agents we tested showed any activity for suppressing or curing the infection.

Bovine immune concentrate has been used successfully to treat rotaviral[37] and *Cryptosporidium parvum*[16] infections in the small bowel and colon. It is therefore unlikely that it was inactivated in the stomach.[38] It is more likely that the bovine immune concentrate was unable to penetrate the mucus barrier in sufficient concentrations to have a measurable effect. Relatively high dosages of BIC did not result in a rise in quantitative serum antibovine immunoglobulins.

Oral administration of 3′-sialyllactose did not have a measurable effect on the density of the *H. pylori* present, nor on the severity of the inflammatory response. Likewise, neither did the high dose or the low dose of rhLf show either a suppressive or a beneficial effect regarding the elimination of *H. pylori* infection. It remains unclear if a peptide fragment of Lf, lactoferricin B, which has been shown to have potent *in vitro* antimicrobial activity,[39] would be efficacious *in vivo*.

The experiments were designed to test what was believed to be the maximum practical doses of each agent. Therefore, the possibility of administration of higher doses to assess efficacy is unlikely to produce a better result. Perhaps larger doses, a longer duration, concomittant acid-suppressing therapy or a combination of these approaches may be needed if a beneficial effect is to be seen.

An improved understanding of the delivery of the drug(s) to the micro-environment of the *H. pylori* infection is required if xenobiotic medications such as bovine immune concentrate are to be used effectively. One role of the novel therapies may be to enhance the effectiveness of antimicrobial therapy. Our bias is that for an agent to be a successful adjuvant, either it must have some inherent antibacterial activity[40] or have a defined rationale (e.g. mucolytics used to uncover the *H. pylori* bacteria.[41] The agents used in this study have a rational basis and subsequent experiments will need to evaluate new parameters and combinations if they are ultimately to have a role in *H. pylori* therapy.

ACKNOWLEDGEMENTS

This work was supported in part by the generous support of Hilda Schwartz, by Galagen Inc., Neose Technologies Inc., and Agennix Inc., who supplied the test materials and by Enteric Products Inc. who kindly donated reagents for serological testing. A portion of this work was supported by NIH Grant M01-RR00188 (General Clinical Research Center).

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6

DUPLICATE

FOR IMMEDIATE RELEASE

Agennix Receives Broad Patent Covering Production of Human Lactoferrin in Eukaryotic Cells

Houston, TX -- May 10, 2001 -- Agennix, Incorporated, a developer of protein and peptide-based drugs targeting oncology, infectious disease and dermatology, today announced the issuance of a patent broadly covering the method for expressing human lactoferrin in eukaryotic cells. The patent, U.S. Patent No. 6,228,614, broadens the Company's previous patent coverage to include a greater variety of potential production systems for this protein. The patent is assigned to the Baylor College of Medicine and is licensed exclusively to Agennix. The issuance of this patent brings to Agennix 13 U.S. patents in its growing portfolio. Corresponding patents have now been granted in many countries throughout the world..

"This patent significantly expands our patent coverage of recombinant human lactoferrin," said Richard Barsky, CEO of Agennix. "The issuance of this patent confirms our view that Baylor scientists have made substantial contributions in the field of lactoferrin. These contributions are now being recognized by patent offices worldwide."

The inventors on the patent are Drs. Orla Conneely, Bert O'Malley, both on Agennix's Scientific Advisory Board, and Denis Headon, President and Chief Scientific Officer of Agennix.

Agennix is a privately-owned biopharmaceutical company focused on research and development of recombinant human lactoferrin (rhLF), a natural and safe anti-infective and anti-inflammatory protein, and a variety of related peptides. Holding 40 issued patents and 84 pending patents, the Company is engaged in programs that address large market opportunities and unmet medical needs in the areas of oncology, infectious disease and dermatology. Agennix has completed numerous pre-clinical and clinical trials with rhLF demonstrating the enormous potential of lactoferrin in a wide range of clinical conditions. More information about Agennix is available on the Company's web site at: www.agennix.com.

Agennix Incorporated

OU

REC'D AUG 0 3 2005

July 29, 2005

REC'D AUG 0 2 2005

Lester M. Crawford, D.V.M., Ph.D. (HF-1)
Commissioner of Food and Drugs
U.S. Food and Drug Administration
Department of Health and Human Services
Room 1471
5600 Fishers Lane
Rockville, MD 20857

RE: Ventria Bioscience Petition for GRAS Status for Recombinant Human Lactoferrin Purified From Rice

Agennix

Dear Dr. Crawford,

Since leaving the FDA, I've done a variety of things and one of those things is serving as Chairman of the Board of Agennix, Inc. We would like your guidance, advice and help on a regulatory matter about which we have serious concerns – Ventria Bioscience's GRAS petition No. 000162 with the FDA's Center for Food Safety and Applied Nutrition (CFSAN), requesting GRAS status for recombinant human lactoferrin (rhLF).

With FDA guidance, Agennix has been developing this recombinant protein as a pharmaceutical drug under FDA INDs since 1996. (See, e.g., IND No. 6799, IND No. 11728 and IND No. 8546) We have expended significant resources – over $70 million to date – to try to demonstrate to FDA/CDER satisfaction the safety and efficacy of rhLF in indications such as renal cell carcinoma, non-small cell lung cancer and diabetic foot ulcers. We have conducted Phase II clinical trials in the latter two indications that achieved their primary endpoints, and hope to start Phase III trials this year.

We believe that Ventria's approach – seeking GRAS status from CFSAN for this novel, biologically active compound to avoid the more stringent drug approval requirements under CDER – undermines established FDA procedures for drug approval, and disincentivises drug development for indications like graft vs. host disease (for which Agennix rhLF has been granted Orphan Drug status) and diabetic foot ulcers (for which Agennix rhLF has been awarded Fast Track status).

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Orphan Drug status is supposed to provide exclusivity for seven years after marketing approval to the developer of a drug so that drug developers can justify the cost of development for disease indications that may be very serious but not very common. However, such "exclusivity" would be effectively eliminated if other companies were granted GRAS status for the same compound and could freely sell it to the same patients without having incurred the expense of clinical trials to demonstrate either safety or efficacy, and without the expense of producing to cGMP standards.

We therefore request that you ensure that FDA/CDER coordinate closely with CFSAN and resolve this potential regulatory inconsistency by ensuring that the GRAS petition for rhLF is denied.

Ventria has incorrectly asserted that rhLF has been demonstrated to be safe for general usage, has a long history of human use, is substantially equivalent to native human lactoferrin, and is intended for use as a food ingredient. We have already written to CFSAN (with supporting documentation) refuting these assertions.

RhLF is a novel compound. When Agennix requested a USAN name for our rhLF product, the USAN Council, based on guidance from CBER, responded that the USAN name would need to reflect the unique identity of the Agennix recombinant lactoferrin. Specifically, this name was required to include a prefix reflecting the particular amino acid sequence of the recombinant protein and a suffix reflecting the unique glycosylation pattern, which is different from that found in native human lactoferrin. Based on these considerations, USAN assigned Agennix's recombinant human lactoferrin the unique name of "talactoferrin alfa."

Both of these considerations would also apply to recombinant human lactoferrin produced in rice – namely, a specific amino acid sequence, and a glycosylation pattern different from that found in native human lactoferrin. Thus, Ventria's rhLF is a novel molecule that is being actively developed as a drug (see below), and would appear to clearly fall within the regulatory purview of CDER.

Ventria's attempt to obtain GRAS listing through CFSAN raises important policy issues within the FDA that should be addressed:

- Should biologically active recombinant proteins, with known therapeutic potency in the treatment of disease and without a previous record of broad human consumption, be held to a lower standard for demonstration of safety than other drugs seeking approval at the FDA simply because small quantities of a native analogue of that protein (with different glycosylation and possible differences in amino acid sequence) exist endogenously in humans?

- Should companies be allowed to circumvent the established regulations for drug development of such biologically active proteins (which are already in clinical trials under CDER approved INDs) simply by calling them "food ingredients," while publicly proclaiming their medicinal value?

- Should "medicinal" foods (foods containing pharmacologically active ingredients) be evaluated under the food regulations using GRAS status or food additive petitions rather than the drug and biologic regulations?

We believe, for the reasons below, that any CFSAN GRAS authorization of Ventria's rhLF for use in food would create two wholly inconsistent safety standards and regulatory pathways for drug approval at the FDA:

1. Notwithstanding claims to the contrary, Ventria is, in fact, developing and promoting rhLF as a pharmaceutical drug for the treatment of human disease.

2. RhLF has been in clinical development by Agennix under multiple FDA INDs since 1996, and CFSAN should not be a backdoor to approval of drugs already in development at CDER.

1. Ventria's claim in its GRAS petition that rhLF is a food ingredient is subterfuge.

Ventria has sought GRAS status for rhLF under CFSAN regulations claiming that rhLF is a food ingredient suitable for broad, unregulated human consumption. Ventria's own public statements, however, contradict this claim and demonstrate the Company's real intention to promote rhLF as a pharmaceutical drug for the treatment of human disease.

In testimony on June 29, 2005, before the United States House of Representatives Subcommittee on Rural Enterprises, Agriculture, and Technology, Ventria stated that the company is engaged in developing plant-made pharmaceuticals and biologics intended as medicines: "Ventria Bioscience is a plant-made ***pharmaceutical*** company that utilizes rice and barley as a factory to produce biologic products," and "Ventria believes this technology will lead to more affordable ***medicines*** for a much broader ***patient population*** than what is possible with conventional biopharmaceutical production technology today" **(Appendix 1)**.

Ventria also presented, in this recent testimony, medical claims for its rhLF, branded as Lactiva™, stating, "***Ventria believes it [Lactiva] can improve the recovery rate and reduce the severity or duration of diarrhea***" in children, and Ventria is targeting "***Inflammatory Bowel Disease***" and "***Chronic Lung Infections caused by***

Pseudomonas" in patients with Cystic Fibrosis **(Appendix 1)**. This last statement was made despite the fact that a recently published scientific study has implicated Ventria's own lactoferrin in the induction of antibiotic resistance to Pseudomonas in patients with Cystic Fibrosis **(Appendix 2)**. Ventria has made many other public statements characterizing their activities as pharmaceutical drug development **(Appendix 3).**

Not only is Ventria talking about the drug properties of rhLF before Congress, on its website and in the press, Ventria has even published an article on rhLF's efficacy as an antibiotic comparable to approved antibiotic drugs **(Appendix 4)**. Further, Ventria appears to have conducted clinical trials, both within the U.S. and in South America, to evaluate the use of rice-derived rhLF as a drug to treat anemia and diarrhea **(Appendix 3).**

It is obvious that recombinant human lactoferrin's effects as a drug are the reasons Ventria is seeking to produce it and will be the reason people purchase it, and that its use in food would provide a back-door route to marketing approval for a novel pharmaceutical agent.

Agennix

2. Recombinant human lactoferrin is a drug, has been regulated as a drug, and should continue to be regulated as a drug.

Recombinant human lactoferrin is a drug and has been in clinical development as such by Agennix since 1996. ***Even Ventria promotes rhLF (on its website, in published presentations and in press releases) as a drug intended to be used to treat acute diarrhea, acute respiratory infections, topical infections, fungal infections, anemia and inflammation*** **(Appendix 3)**.

Agennix obtained its first IND for oral rhLF in 1996 (IND 6799) and its dermal IND (IND 8546) in 1999. With the advice and guidance of FDA/CDER, Agennix has spent over $70 million developing rhLF and hopes to begin Phase III trials this year in non-small cell lung cancer and diabetic neuropathic ulcers. Over 440 people have been treated to date with Agennix's oral rhLF (at centers including M.D. Anderson, Stanford, University of Chicago, UCLA, Cleveland Clinic and Baylor) and we are confident that, together with our collaborators, we have generated and published far more data on recombinant human lactoferrin than Ventria. However, even Agennix would not claim to have come close to establishing the level of safety required for a GRAS listing of this biologically active recombinant protein, and Agennix has not yet been permitted by the FDA/CDER to market recombinant human lactoferrin.

Agennix has been following all of the FDA guidelines and requirements for drug development for many years. CFSAN should not be a back door to approval of drugs already in development at CDER. That would unfairly disadvantage those diligently

following appropriate drug development procedures, disincentivise drug development (especially for orphan indications) and create two wholly inconsistent safety standards and regulatory pathways for drug approval at the FDA.

This matter is of paramount importance to Agennix. We would, therefore, like to follow-up with you in person to discuss what actions might be taken to ensure that GRAS status is not inappropriately granted for rhLF. We would welcome participation of both CFSAN and CDER officials at such meeting. Ventria's GRAS petition is currently pending at CFSAN and we believe that some urgency is needed in addressing this matter.

I will be contacting you in the near future to arrange a convenient time when we might have such a discussion. We sincerely appreciate your attention to this situation and look forward to talking with you.

Sincerely yours,

Frank E. Young, M.D., Ph.D.
Chairman of the Board of Directors
Agennix, Inc.

Cc: Janet Woodcock, M.D., Acting Deputy Commissioner for Operations
Robert Brackett, Ph.D., Director, CFSAN
Laura Tarantino, Ph.D., Director, Office of Food Additive Safety, CFSAN
Steven Galson, M.D., Ph.D., Acting Director, CDER
John Jenkins, M.D., Director, Office of New Drugs, CDER
Richard Pazdur, M.D., Director, Division of Oncology Drug Products, CDER
Patricia Keegan, M.D., Dir., Div. of Therapeutic Bio. Oncol. Products, CDER
Karen Weiss, M.D., Director, Office of Drug Evaluation, CDER
Marc Walton, M.D., Dir., Div. of Therapeutic Bio. Int. Med. Products, CDER
Sheldon Bradshaw, Esq., Chief Counsel, Food and Drug Division
Marlene Haffner, M.D., M.P.H., Director, Office of Orphan Products Dev.

Attachments

Agennix Incorporated

September 11, 2006

Laura M. Tarantino, Ph.D. (HFS-200)
Director, Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
Room 3044
University Station
4300 River Road
College Park, Maryland 20740

Agennix

Re: Safety Concerns Raised by Recombinant Human Lactoferrin from Rice
(GRN No. 000162 Submitted by Ventria Bioscience)

Dear Dr. Tarantino:

I am writing with further information concerning GRN No. 000162 submitted by Ventria Bioscience ("Ventria"). We have asked a group of scientific experts to review the May 9, 2006 submission by Ventria to your office, which we obtained under the Freedom of Information Act. In that submission, Ventria responded to a series of questions that your office posed to Ventria. Based on the conclusions reached by our experts, we believe more than ever that the GRAS criteria have not been satisfied.

Attached you will find, under a cover letter from Atul Varadhachary, M.D., President and Chief Operating Officer of Agennix, Inc., a comprehensive scientific analysis of Ventria's responses to your office's questions, as well as two additional supporting letters from scientific experts. Those experts conclude, unequivocally, that significant safety issues exist and important unanswered questions remain with respect to Ventria's GRN for its rice-derived recombinant human lactoferrin (rhLF).

Accordingly, as before, we continue to believe that Ventria has failed to meet all three prongs of the statutory criteria for GRAS status:

1. Insufficient Technical Evidence of Safety. In reviewing Ventria's response to CFSAN's most recent questions, our experts have concluded: "The data and the published opinion clearly show that there is an undeniable risk of breakdown of immune tolerance and of autoimmunity with oral consumption of rice-derived rhLF, with potentially serious

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consequences." This complements and reinforces the many scientific points made by our experts in our earlier submission questioning the scientific evidence advanced by Ventria. As the law incorporates the rigorous standard of Ventria needing to demonstrate a "reasonable certainty of no harm," we believe the record shows, over and over, that Ventria has failed to meet its legal burden.

2. Lack of Publicly Available Data. As described in our previous submissions, Ventria lacks data in the public domain to substantiate many of their key points.

3. Severe Conflict Among Experts. Our current submission continues to document that a severe conflict exists among experts in the field. Indeed, it is hard to imagine experts being more apart in their scientific opinions. The experts that we have identified, and who have reviewed the materials submitted by Ventria, are unanimous in their belief: (a) that safety of rice-derived rhLF has not been established; (b) that important safety concerns have been raised based on existing information; and (c) that extensive clinical testing is needed to establish the safety of Ventria's rice-derived rhLF for long term use in humans, particularly for use by vulnerable populations in settings without medical supervision.

Taken together, we believe that CFSAN should conclude that GRN 000162 does not meet the standards for being Generally Recognized as Safe for use in food.

Thank you, as always, for your careful and thorough review of these issues. Please let us know if we can provide additional information or clarification.

Sincerely yours,

Rick Barsky

Rick Barsky
Chief Executive Officer

Attachments

cc: Robert Merker, Ph.D. (HFS-255)
Consumer Safety Officer
Division of Biotechnology and GRAS Notice Review

Agennix

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Agennix Incorporated

September 11, 2006

Laura M. Tarantino, Ph.D. (HFS-200)
Director, Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
Room 3044
University Station
4300 River Road
College Park, Maryland 20740

Re: Safety Concerns Raised by Recombinant Human Lactoferrin from Rice
(GRN No. 000162 Submitted by Ventria Bioscience)

Dear Dr. Tarantino:

We are writing with further information concerning GRN No. 000162, submitted by Ventria Bioscience ("Ventria" or "Notifier"). We recently received a copy, under the Freedom of Information Act, of the Ventria's submission dated May 9, 2006 in response to questions posed by CFSAN regarding the potential immunological health risks of recombinant human lactoferrin (rhLF) produced in rice. We believe that the questions asked by the Agency are both relevant and important. We are concerned that Ventria's response did not fully provide or accurately characterize the data relevant to the questions asked by CFSAN. Therefore we are providing below a more comprehensive scientific response to your concerns.

In preparing this response, we consulted with several highly qualified scientists and physicians who are scientific experts in the fields of immunology, glycobiology, and orally administered immunomodulatory agents. Their conclusions stand in sharp contrast to the positions advanced by Ventria. Indeed, our experts have concluded:

- The data and the published expert opinion clearly show that there is an undeniable risk of breakdown of immune tolerance and of automimmunity with oral consumption of rice-derived rhLF, with potentially serious consequences.

- These risks may be even higher in susceptible populations, including children, immunocompromised individuals, those with pre-existing autoimmune disease, and those with gut pathology.

- Orally administered rhLF has broad systemic immunomodulatory effects with long-term consequences that have not been evaluated.

- There does not appear to be any published data on sustained human consumption of high-doses of human glycoproteins with plant-type glycosylation.

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These conclusions reinforce our earlier position that Ventria has not—by a wide margin—established scientific evidence of safety as would be needed to meet the rigorous criteria needed for GRAS status.

Accordingly, please find attached a scientific paper signed by Richard D. Cummings, Ph.D, Arno Kromminga, M.D., Federica Pericle, Ph.D. and Michael P. Sherman, M.D. In addition, attached are supplementary letters from Margalit Mokyr, Ph.D. and from Dr. Sherman. The CVs for Dr. Cummings and Dr. Kromminga are already on file with you through our earlier submission. The CVs for Dr. Mokyr, Dr. Pericle and Dr. Sherman are attached.

Please note that the attached paper refers to confidential research conducted by Agennix. This information is available to the agency in our IND on file with FDA's Center for Drug Evaluation and Research (CDER). Please let us know if you need assistance in locating this information in our IND file.

We appreciate CFSAN's consideration of this important information while the Ventria's GRAS notification for rhLF from rice is considered. Please do not hesitate to contact us if there are any questions or if additional information would be useful.

Agennix

Sincerely yours,

Atul Varadhachary.

Atul Varadhachary, M.D., Ph.D.
President & Chief Operating Officer

Attachments

Cc: Robert Merker, Ph.D. (HFS-255)
Consumer Safety Officer
Division of Biotechnology and GRAS Notice Review

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RESPONSE TO QUESTIONS POSED BY CFSAN: GRAS NOTICE NO. 000162 SUBMITTED BY VENTRIA BIOSCIENCE

This paper analyzes a submission by Ventria Bioscience ("Ventria" or "Notifier") submitted to the Center for Food Safety and Applied Nutrition (CFSAN), Food and Drug Administration (FDA), dated May 9, 2006, which was submitted by Ventria in response questions posed by CFSAN. Ventria's submission, a copy of which was obtained under the Freedom of Information Act, indicated that CFSAN had asked the following three questions:

1. Whether the consumption of large amounts of recombinant human lactoferrin would result in the possibility of a breakdown of tolerance?"
2. Whether "consumption of large doses of lactoferrin, particularly lactoferrin that differs slightly from the endogenous form with respect to small sequence or conformational differences, could theoretically act as a trigger to autoimmune responses for those genetically predisposed?"
3. "Is there published data on other products that would address the consumption of other human (or relevant model animal) N-linked glycoprotein produced in plants that have been consumed by humans (or those animals) at high levels?"

These questions are addressed in order below.

1. Breakdown of Immune Tolerance. The potential risk of a breakdown of immune tolerance is an important concern. The induction of anti-lactoferrin antibodies could have serious consequences by "neutralizing" many other vital functions of endogenous lactoferrin, which is a degranulation product of neutrophils involved in, among other things, the regulation of inflammation and protection against the development of cancer. Anti-lactoferrin antibodies could also compromise the efficacy of exogenously administered rhLF following its potential future approval as an anti-cancer drug. The Notifier's response does not appear to alleviate concerns about this potential risk. Our understanding of the Notifier's argument can be summarized as follows:

a. Orally administered proteins containing soluble antigens tend to be tolerogenic rather than sensitizing, as demonstrated by the lack of orally effective vaccines.

b. Further, large doses of orally administered proteins tend to be tolerogenic rather than sensitizing. In any case, there is no definitive evidence that oral lactoferrin crosses the adult human gut mucosa in large quantities, so it is unlikely to encounter the immune system in adult humans with healthy guts.

c. Furthermore, the systemic immune effects observed in patients receiving oral lactoferrin are modest relative to those observed in animal models.

d. While numerous studies have demonstrated that glycosylated epitopes are extremely immunogenic and can cause a breakdown of tolerance, "there is no evidence in the

literature that would support that antigen processing would be influenced by minor differences in glycan structure".

The Notifier concludes that "at the proposed intake levels, the response is more likely to be in support of oral tolerance". Thus, the Notifier suggests that despite strong indications that a breakdown of immune tolerance can be a significant risk in this context, and despite the lack of clinical evidence that rice derived rhLF causes immune tolerance, this risk should be ignored as part of the GRAS evaluation.

Even if the facts were as described by the Notifier, we would disagree with the Notifier's conclusion. When there is supporting data for a clearly articulated risk with potentially major health consequences, particularly for underage and vulnerable populations, the onus must be on the GRAS applicant to disprove (through animal studies and human clinical trials of appropriate size and duration) the apparent risk. Any attempt by the Notifier to shift the burden of proving potential harm to the Agency should be rejected. Before making rhLF available in unlimited quantities without any medical supervision, it would be prudent and appropriate to evaluate the immunological consequences in controlled human trials.

However, as discussed further below, we do not believe that the facts articulated by the Notifier are complete and accurate. We believe the Notifier significantly understates the risk of a breakdown of tolerance.

1a. Immunological Effects of Orally Administered Proteins. The Notifier asserts that oral administration of "soluble antigens lead to systemic tolerance" and that oral glycoproteins such as lactoferrin are not taken-up by the Microfold cells (M cells) of the Peyer's patches, which is the major inductive site for gut associated lymphoid tissue (GALT) immune responses. Although many orally administered antigens will be tolerogenic, it is certainly not true in all cases. Further, the Notifier's assertion that "[s]oluble proteins and glycoproteins are not taken up by M cells" (page 2, paragraph 1) is inconsistent with the literature (Chabot 2006, Favre, 2005, Lo 2003). Studies using OVA, which the Notifier cites as an example of an orally tolerogenic protein (page 2, paragraph 2), have shown that a cytotoxic T lymphocyte response can be induced by oral administration of OVA antigen and indeed trigger the onset of autoimmune disease (Blanas 1996, Blanas 1999). Substantial CD8+ cytotoxic T- lymphocyte (CTL) responses were observed in mice fed with OVA, leading the investigators to conclude that "the intragastric route of antigen administration does not necessarily provide a default mechanism for tolerance induction". Proliferation of both OVA-specific CD8+ lymphocytes and CD4+ lymphocytes was observed in the Peyer's patches and mesenteric lymph nodes two days after feeding OVA to mice (Blanas 2000). Even more significantly, feeding exogenous OVA to transgenic mice expressing OVA as a pancreatic autoantigen led to the induction of autoimmune diabetes in about half the animals. Multiple OVA feedings further increased the incidence of autoimmune diabetes, leading the investigators to conclude that "feeding antigen can activate autoaggressive CTLs" (Blanas 1996). This situation is highly relevant to the Notifier's situation -- an exogenous version of a normally self-protein, derived from a different host, triggering a strong autoimmune response with major health consequences.

In one of several other published examples, De Weerd et al looked at the ability of an orally administered recombinant version of a pollen allergen to induce a systemic immune response (de Weerd 2003). They found a robust systemic response including "the induction of both blocking and interspecific cross-reactive antibodies".

The Notifier also comments that induction of tolerance is "classically associated with a decrease, not an increase, in pro-inflammatory cytokines. This is correlated with the production of anti-inflammatory/immunosuppressive cytokines such as TGFβ and IL10" (page 4, paragraph 2). The Notifier does not provide or reference any data suggesting that oral administration of rhLF is associated with anti-inflammatory/immunosuppressive cytokines. In fact, published data in both mice (Varadhachary et al) and humans (Hayes et al) with Agennix's rhLF shows that oral rhLF leads to the up-regulation of the pro-inflammatory cytokine IL-18.

In support of their contention that orally administered proteins tend to be non-immunogenic, the Notifier asserts a lack of orally active vaccines (page 2, first paragraph). However, contrary to this assertion, numerous orally active vaccines have been developed in recent years, including polio, typhoid, cholera, *E. coli*, Norwalk virus and rotavirus vaccines (Case Western Reserve University). This remains a very active area of research and development with even a number of different transgenic plant species being developed as a means of delivering oral vaccines (Streatfield 2006, Giudice 2006, Han 2006, Duke Human Vaccine Institute, Karaman 2006).

1b. Immunological Processing of Oral RhLF. The Notifier argues that large doses of orally administered proteins tend to be tolerogenic but also argues that oral rhLF does not cross the adult human gut mucosa, and is unlikely to encounter the immune system in adult humans with a healthy gut. These arguments are internally inconsistent, and are also inconsistent with the available data.

Dendritic cells (DCs), which are specialized for antigen presentation play a critical role in initiating the adaptive immune response (Cella et al, 1997). It is well known that DCs interdigitate between the gut enterocytes and sample luminal contents, including soluble proteins (Neiss 2005). Thus, even in the absence of any transport across the gut lumen, orally administered rhLF will be in contact with the immune system.

However, there is published data demonstrating that orally administered lactoferrin crosses the gut mucosa in animal models (Harada 2003, Muri 2005, Talukder 2003). The extent of uptake is even higher in immature animals and in settings with a compromised gut mucosa. The Notifier asserts that these findings are inapplicable to humans without providing any basis for that assertion. In confidential animal work, Agennix showed that although rhLF can be detected in the intestinal Peyer's Patches (PP) following oral administration, it is not detectable in systemic circulation, even using sensitive ELISA assays. Similarly, in humans, there is no basis for expecting to be able to detect rhLF in circulation even after it crosses the gut mucosa. Detection sensitivity in humans is further reduced by the background signal from endogenous circulating lactoferrin (Levay 1995).

In the confidential Agennix research described above, orally administered rhLF was found in mouse Peyer's Patches (PP), both as demonstrated by ELISA analysis of the PP and by FACS analysis of immune cells within the PP. RhLF binding was detected on 15% to 50% of the immune cells examined in the PP. The total amount of rhLF found in the PP was low (reaching a peak of approximately 0.05 mg/mL rhLF within the PP following dosing with a 100 mg/mL rhLF). The finding that orally administered rhLF is taken up in small quantities and binds to immune cells in the PP contradicts both assertions made by the Notifier: (i) that oral lactoferrin does not cross the adult gut mucosa (page 2, paragraph 3, line 12), and (ii) that rhLF consumption will be "far in excess of [the dose] required for high dose or deletional tolerance" (page 1, last paragraph). Further, the Notifier acknowledges that oral rhLF crosses the gut mucosa in children and in individuals with intestinal pathology, which is relevant to the GRAS filing since children are included in the target population, with child-specific products such as popsicles and oral rehydration solution.

1c. Systemic Immunostimulation by Oral RhLF. The Notifier acknowledges that oral rhLF can induce a strong systemic immune response in animal models but makes the assertion that any systemic immunostimulation in humans is likely to be modest. This assertion is not supported by the available data. The Notifier cites a study where oral administration of bovine lactoferrin to HIV positive children produced "only a very modest immunological effect" with circulating CD4+ lymphocytes increasing from 21.5% to 25.4%. CD4+ counts are expected to drop in untreated HIV positive individuals, so oral lactoferrin's immunostimulatory effect is likely to be understated in this trial. Further, the study used a heterologous protein, bovine LF (bLF) in humans, which has only a 68% amino acid sequence homology to rhLF (Lonnerdal, 1995). BLF is likely to be less effective than rhLF in humans due to its lower binding affinity to human LF receptors (Kawakami, 1991) and its far greater susceptibility to gastric digestion (van Veen, 2004). But even without these considerations, the cited immunostimulatory effect is substantial, amounting to an almost 20% relative increase. Given the large absolute numbers of circulating CD4+ cells and their important role in modulating the immune system, even high single-digit relative increases would be considered clinically significant in these patients.

The Notifier also cites a study with Agennix's rhLF (Hayes 2005), stating that treatment of cancer patients with oral rhLF "gave only a 15% increase in circulating IL18". In animal experiments with Agennix's rhLF, oral rhLF stimulated the production of IL-18 when measured either in circulation or in the gut (Varadhachary 2004). Oral rhLF also demonstrated systemic immunostimulation as well as anti-cancer activity in this work, with tumor growth inhibition comparable to that observed with approved anti-cancer. Consistent with rhLF's primary site of action in the Gut Associated Lymphoid Tissue (GALT), IL-18 increases in the gut were up to eight times higher than those observed in circulation (unpublished data). Thus the increases in circulating IL-18 observed in cancer patients by Hayes et al are consistent with the even more substantial immunostimulation expected in the GALT. Further, confidential data from that same trial also showed increases in other indicators of immunostimulation through cytokine and FACS analysis. An apparent anti-cancer activity was also observed in patients with non-small cell lung

cancer and with Renal Cell Cancer (unpublished) who had failed previous anti-cancer therapy.

The potential risks from immunostimulation are significant, as was recently seen in the tragic case of TGN1412 (TeGenero Immuno-Therapeutics). TGN1412 is an immunomodulatory antibody that (like rhLF) stimulates T-cell expansion and activation. Despite a strong preclinical safety record, including testing in primates, TGN1412 produced severe and unexpected reactions in a Phase I clinical trial involving seven healthy volunteers. On March 13, 2006, six of the volunteers who received the active drug suffered organ failure and violent, life-threatening side-effects. While this is an extreme case of acute immune reaction, it suggests a need for caution with immunomodulatory agents, especially with compounds that will be administered for extended periods of time.

1d. Effect of Changes in Glycosylation. While acknowledging that numerous studies have demonstrated that glycosylated epitopes are extremely immunogenic and can cause a breakdown of tolerance, the Notifier asserts that "there is no evidence in the literature that would support that antigen processing would be influenced by the minor differences in glycan structure". This assertion is inconsistent with the published literature that explicitly discusses the concern that modifications in the glycosylation of transgenically expressed human glycoproteins can result in immunogenicity (Bakker 2006). Changes in glycosylation can influence antigenicity and may affect several parameters of the immune response, including antigen processing and the type of immune response elicited. We discuss three relevant publications below:

- Backlund et al evaluated the role played by glycosylation in immune tolerance (Backlund 2002). The investigators used a human DR4-transgenic mouse model to determine the impact of glycosylation on the induction of CII specific T cell tolerance. An important conclusion of the study is that "in DR4-transgenic mice expressing huCII, and most likely also in humans, T cells recognizing the nonglycosylated 263–270 epitope are strongly tolerized, or even deleted, whereas T cells specific for the different glycosylated peptides persist." Beyond looking at the presence or absence of glycosylation, the investigators also evaluated the effect of relatively minor changes in the carbohydrate structure. In addition to nonglycosylated peptides and peptides glycosylated with galactose, they synthesized peptides glycosylated with 2-deoxy, 3-deoxy or 4-deoxy galactose. Each entity yielded a distinct hybridoma response profile, demonstrating that even minor changes in the carbohydrate structure have a substantial impact on immune recognition. As pointed out by the investigators, "the relevance of the findings made in mice expressing huCII and DR4 is evident because 30% of the investigated RA patients exhibited a predominant response to the glycosylated forms of the CII263–270 epitope. The investigators go on to conclude that their findings "show that the physiological post-translational modification of variable carbohydrate attachment converts the immunodominant naked self-peptide, which is tolerogenic, into several cryptic self-determinants that remain immunogenic."

- Gonzalez et al evaluated the effect of conformational changes including glycosylation, and protein microheterogeneity on the immunogenicity of Ole e 1, the

major olive allergen (Gonzalez 2002). The relevance of glycosylation was examined using not only chemically deglycosylated protein, but also using recombinant versions of the proteins purified from a heterologous expression system. The differentially glycosylated proteins showed distinct patterns of recognition by Ole e 1-specific monoclonal antibodies.

- Prescott et al describe a study that demonstrated that expression of a recombinant protein in a different host resulted in a protein with altered glycosylation and increased immunogenicity (Prescott 2005). Specifically, the expression of one plant protein (bean alpha-amylase inhibitor; aAI) in a different plant species (peas) resulted in a protein with an unaltered primary amino acid sequence but with a change in protein glycosylation. Although oral consumption of the native bean form of aAI did not promote immunological responsiveness or inflammation in mice, oral consumption of the transgenic aAI did induce aAI-specific systemic immunological responsiveness as measured by a variety of parameters including IgG titers, DTH responses, and TH2-type pulmonary inflammation. Furthermore, co-administration of the transgenic aAI with other dietary proteins promoted immunological cross priming and elicited immunogencity to unrelated dietary proteins. The study demonstrates the structural and immunological consequences that can result from even relatively small changes in glycosylation that occur even in relatively closely related species such as two plants.

  This transgenic pea strain had been in development by the Australia-based Commonwealth Scientific and Industrial Research Organization (CSIRO) for almost ten years prior to the work by Prescott et al. To create an insect resistant pea strain, scientists at CSIRO took a defense gene from the closely related kidney beans and transferred it to peas. The transgenic strain was tested in animal models for several years with no indication of safety concerns. A published rat feeding study where the animals received the transgenic pea strain at doses up to 650 g/kg concluded that the protein was safe (Pusztai 1999). However following full molecular characterization of the molecule, including identification of the minor glycosylation differences, Prescott et al conducted the work that identified the protein's immunogenicity and its major safety concerns. Following publication of this work, CSIRO terminated all testing of the transgenic pea strain in late 2005 (GMO Compass).

- Finally, the Notifier rejects the concept of "hidden epitopes" as controversial. However, even the review article cited by the Notifier, Anderton specifically considered this issue and concluded that "post-translational modifications (PTMs) of mammalian proteins, including glycosylation can allow immune recognition of neo-self epitopes. ... These effects can vary from overt increase in affinity of MHC or T-cell receptor binding, to more subtle effects on the activity of proteolytic enzymes involved in antigen processing." (Anderton 2004) It does not appear that the appearance of antibodies to hidden epitopes was tested in any of the published studies with the Notifier's rhLF. Further, considering that rhLF would be a heterologous protein in animal models, the appearance of hidden epitopes could only be tested in an appropriately sized human clinical trial, with the subjects dosed for an immunologically relevant period of time (e.g. 12 months), and powered to detect

even a low incidence of the emergence of hidden epitopes.

2. Risk of Triggering Autoimmunity. As pointed out by CFSAN, considering that rhLF differs from endogenous lactoferrin, and further considering that autoantibodies to lactoferrin may already be present in patients with autoimmune diseases, the risk of inducing or exacerbating automimmunity by oral administration of rhLF needs to be carefully evaluated. There is a large body of literature describing an association between auto-immune disease and auto-antibodies to lactoferrin (Ohana 1998, Roozendaal 1999, van der Woude 1985, Locht 2000, Galeazzi 1998, Okazaki 2000). The Notifier has not fully addressed this issue in its submission.

- The Notifier acknowledges the presence of anti-lactoferrin autoantibodies in human subjects but argues that these may be of relatively low affinity. This conclusion is based on an unpublished study that showed that anti-lactoferrin antibodies from some patients did not interfere with iron binding, without considering the possibility that the antibody recognizes an epitope that is distant from the iron-binding site.

- The Notifier argues, without providing any supporting data, that differences in the polypeptide sequence of orally ingested rhLF relative to the subject's native lactoferrin are of no immunological significance. However, contrary to the Notifier's unsupported assertion, research by a number of groups suggests that even slight changes in protein structure can reveal new epitopes that can trigger immunity. Anderton discusses the minor structural changes resulting from post-translational modifications (PTMs) including amino acid modifications and conversions. This is of particular concern when neo-epitopes from a modified protein are exposed during cell stress, inflammation, or infection. Anderton concludes that "differential PTMs might therefore provide a means of provoking an autoaggressive immune response as a result of infection." (Anderton 2004). Gonzalez et al looked at the effect of 3-D structure on immunogenicity, including variations in protein sequence of Ole e 1, the main olive allergen (Gonzalez 2002). They found that "[t]he IgE from sera of olive-allergic patients showed a significant diversity of binding capacity to the members of the Ole e 1-like family due to the microheterogeneity of their polypeptide sequences, in spite of their highly conserved primary structures."

- The Notifier acknowledges that there is an "extensive literature on autoimmunity to lactoferrin," but argues that these references do not provide definitive evidence that oral rhLF can trigger or exacerbate autoimmunity since they may not exactly represent the clinical setting where individuals consume rice-derived rhLF. In a situation such as this, where there is strong cause for concern but no definitive evidence, a reasonable approach to establishing rhLF's safety would include conducting appropriate clinical trials in individuals at risk, such as those with an autoimmune condition. Although there would be ethical challenges to designing and conducting such a trial in the absence of an expected benefit to these individuals, it would still be far less risky than making the compound available to these individuals in an uncontrolled and unsupervised setting.

- The Notifier does not appear to have directly addressed one of the concerns articulated by CFSAN – particular safety concerns in subpopulations including those with an existing autoimmune condition or those genetically predisposed. Sensitivity to oral antigens varies among people and is dependent on a number of factors. According to Strobel et al

there is "convincing clinical and experimental evidence suggesting that the disturbance of important immunoregulatory and suppressive immunological events induced after oral (mucosal) antigen exposure (oral tolerance) may lead to allergic and autoimmune diseases. The balance between tolerance (suppression) and sensitization (priming) is dependent on several factors" including genetic background and the immunological status of the host (Strobel 2002). Published data also suggests that subpopulations are at risk of an enhanced antibody response to oral antigens. Children with celiac disease have shown enhanced antibody production against dietary antigens, including beta-lactoglobulin in cow's milk (Saalman 2003, Volta 1986). According to the U.S. National Institutes of Health, the incidence of celiac disease is as high as 1 in 133 people and has been on the rise in recent years. Additionally, patients with inflammatory bowel disease (IBD) are known to have increased antibodies to several food and bacterial antigens (Song 1995).

Permitting the distribution of rhLF as GRAS would enable its unsupervised consumption by vulnerable populations including children and individuals with intestinal pathology. Prudence would suggest that adequately powered clinical trials in vulnerable populations should be conducted before even considering making rhLF generally available as a GRAS substance.

3. Other Published Data on Consumption of High Levels of Plant N-Linked Human Glycoprotein.

Although there is an extensive literature on the immunogenicity of carbohydrates found on plant glycoproteins, we agree with the Notifier that there is very limited published data on high level human consumption of N-linked human glycoproteins produced in plants.

In the Notifier's original GRAS submission they state that α(1,3) fucose and β(1,2) xylose glycans appear on virtually 100% of rice-produced rhLF, and that these are cross-reactive carbohydrate determinants (CDDs) known to produce IgE antibodies. These same glycol-epitopes have been shown to induce immunogenicity in other therapeutic proteins produced in plants (Bardor 2002). It has been demonstrated that a considerable number of healthy individuals have antibodies circulating against α(1,3)-fucose and β(1,2)-xylose residues. The important role played by glycosylation in immunogenicity has been discussed previously.

The Notifier cites only two peer-reviewed published studies involving the human consumption of plant-derived human glycoproteins. In the first study (Lonnerdal 2006) only ten women received a single 100 mg dose of iron-saturated rhLF (another ten women received heat-treated rhLF). The only data reported from the trial related to iron absorption and utilization. No data relating to safety or the immunological effects of orally administered rhLF was reported. Further, neither the small dose nor the single-dose regimens are relevant to the proposed GRAS use of rhLF.

The second study cited related to a recombinant antibody made in plants that was applied to the teeth of patients (Ma 1998). 5 µl aliquots were applied directly to the tooth surfaces using a Pipetman and custom made silicone impression trays were inserted "to prevent washing away or dilution by saliva." Thus, this study was focused on the effects of the recombinant protein on the oral cavity and was specifically designed to minimize protein ingestion. Further, as in the other

study cited, the dose administered – 22.5 mg in six doses over three weeks – is far less than the high-dose, long-term consumption of rice-derive rhLF proposed in the Notifier's GRAS application. Thus, neither of the studies cited by the Notifier appear to address CFSAN's question relating to published data on "human (or relevant model animal) N-linked glycoprotein produced in plants that have been consumed by humans (or those animals) at high levels".

Conclusion

In summary, we are concerned that the Notifier's May 9, 2006 response to CFSAN's specific questions was incomplete and does not appear to have characterized the relevant literature accurately. As discussed above, there is significant data in the literature substantiating the concerns expressed by CFSAN in the questions addressed to the Notifier. The data and the published expert opinion clearly show that there is an undeniable risk of breakdown of immune tolerance and of automimmunity with oral consumption of rice-derived rhLF, with potentially serious consequences. These risks may be even higher in susceptible populations, including children, immunocompromised individuals, those with pre-existing autoimmune disease, and those with gut pathology. It is also clear that orally administered rhLF has broad systemic immunomodulatory effects with long-term consequences that have not been evaluated. Further, there does not appear to be any published data on sustained human consumption of high-doses of human glycoproteins with plant-type glycosylation.

Whether concerns about the potential risks of rhLF are justified remains to be determined through competent scientific research. The only means for making such a determination is to conduct robust, controlled human clinical trials of appropriate scope and duration. In the case of rhLF, due to the potentially long periods required to detect the development of immunogenicity (up to one year), this would involve long-term trials involving a large number of subjects.

In view of the documented risks, and until such time as the appropriate clinical data is available, rhLF from rice, or any other source, should not be made available to large portions of the population without medical supervision. Accordingly, it is our view that GRAS status for rhLF is not appropriate at this time.

Respectfully submitted,

Richard D. Cummings, Ph.D.
William Patterson Timmie Professor,
Chair Department of Biochemistry,
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Dr. Arno Kromminga, M.D.
Director, Immunology
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RESPONSE TO QUESTIONS POSED BY CFSAN: GRAS NOTICE NO. 000162 SUBMITTED BY VENTRIA BIOSCIENCE

The preceding response to CFSAN questions posed to Ventria Bioscience concerning recombinant human lactoferrin produced in rice has been provided by and reflects the opinion of:

Signature: 

Name: Dr. Richard Cummings

Title: William Patterson Timmie Professor,
Chair Department of Biochemistry,
Emory University School of Medicine

RESPONSE TO QUESTIONS POSED BY CFSAN: GRAS NOTICE NO. 000162 SUBMITTED BY VENTRIA BIOSCIENCE

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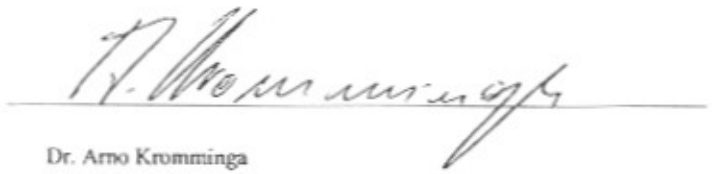
Signature: 

Name: Dr. Arno Kromminga

Title: Director, Immunology
Institute for Immunology, Clinical Pathology, Molecular Medicine (IPM)
Hamburg, Germany

RESPONSE TO QUESTIONS POSED BY CFSAN: GRAS NOTICE NO. 000162 SUBMITTED BY VENTRIA BIOSCIENCE

The preceding response to CFSAN questions posed to Ventria Bioscience concerning recombinant human lactoferrin produced in rice has been provided by and reflects the opinion of:

Signature: 

Name: Dr. Federica Pericle

Title: Associate Vice President, Biotechnology
University of Texas at El Paso
El Paso, Texas

RESPONSE TO QUESTIONS POSED BY CFSAN: GRAS NOTICE NO. 000162 SUBMITTED BY VENTRIA BIOSCIENCE

The preceding response to CFSAN questions posed to Ventria Bioscience concerning recombinant human lactoferrin produced in rice has been provided by and reflects the opinion of:

Signature: Michael P Sherman MD FAAP

Name: Dr. Michael Sherman

Title: Professor of Pediatrics
Southern Illinois University School of Medicine
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References

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Re: Safety Concerns Raised by Recombinant Human Lactoferrin from Rice
(GRN No. 000162 Submitted by Ventria Bioscience)

Dear Dr. Tarantino:

I am an immunologist at the University of Illinois at Chicago with an academic interest in cancer immunology. I am familiar with recombinant human lactoferrin (rhLF), particularly with its immunomodulating activity in cancer. I have had an opportunity to review the submission dated May 9, 2006 by Ventria Bioscience in response to questions from CFSAN, and I would like to submit the following comments.

I would like first to comment on the suitability of designating any rhLF as GRAS. Based on my knowledge of Agennix's experience with rhLF extending over several years, it is very clear to me that orally administered rhLF has a significant systemic immunomodulatory effect. RhLF stimulates the production of a number of cytokines in the gut, affects the biology of the Gut Associated Lymphoid Tissue (GALT), modulates tumor-associated lymph nodes and induces cellular infiltration into distant tumors. Given Agennix's results, which clearly illustrate the immunopotentiating activity of rhLF (talactoferrin) administered orally, I would be hesitant to consider rhLF as a totally innocuous molecule and I would worry about making rhLF available at unlimited amounts without any supervision. The responses of Ventria Bioscience to the potential problems identified by CFSAN do not alleviate my concerns since, in several of Ventria's statements, there are scientific gaps and inconsistencies, as well as problems with the logic of their assertions.

I will focus my comments on Ventria's response to CFSAN's expressed concern regarding the possibility that consumption of large amounts of rhLF could result in a breakdown of tolerance. The inconsistencies and scientific gaps in their response have important implications to their entire line of reasoning.

UIC

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In several places throughout the document, Ventria dismisses the potential risks of rice-produced rhLF with the argument that lactoferrin is not transferred across the gut mucosa in adult humans (e.g., third paragraph on page 2 and middle of the 4th paragraph on page 3 of their submission). This argument is not supported by the data and even appears to be internally inconsistent:

- In other places in the same sections, they discuss the effects of orally administered lactoferrin on the immune system, which would necessitate that lactoferrin would effectively interact with the gut mucosa and would come in contact with important cellular components of the immune system.

- Moreover, the fact that they state that the unlimited use of rice-produced rhLF would result in deletional tolerance indicates that they expect that rhLF would not only cross the gut mucosa, but would do so in relatively large amounts that would, in turn, come in contact with the immune system (e.g. last paragraph on page 1 and first two paragraphs on page 2).

- In my view, Ventria incorrectly interprets some data by other investigators and uses the incorrect interpretation to advance their case. For example, in the 2nd paragraph on page 4, they state that the effects of oral lactoferrin on the immune system are quite modest since oral administration of bovine lactoferrin to HIV positive children produced an increase in CD4+ cell counts from 21.5% to 25.4%. In my view this is an impressive increase in the percentage of CD4+ cells, especially if one is viewing this increase as a response to lactoferrin. In any event, a close to 20% relative increase in the number of CD4+ T-cells is a substantial increase and clearly illustrates that lactoferrin, administered orally to patients, crosses the gut mucosa and affects components of the immune system.

Ventria suggests that oral rhLF could be expected to induce immune tolerance. They do not present any data in support of their assertions, and their arguments in support are not convincing.

- Although Ventria is correct that, in general, a high dose of antigen can induce deletional tolerance (once it comes in contact with the immune system), it is not clear from the document how much rhLF actually crosses the gut mucosa, nor is it clear whether the physical form of the rhLF is soluble or particulate. The amount of antigen that crosses the gut mucosa, as well as the form of the antigen that is present in the gut, has a major impact on whether tolerance or immunization will ensue. In this regard it should be pointed out that, in addition to the M cells mentioned by Ventria that transport particulate antigens across the gut mucosa, there are also dendritic cells that interdigitate between the gut enterocytes and sample luman contents, including soluble proteins.

- Although Ventria is correct in stating that the induction of oral tolerance is classically associated with a decrease rather than an increase in pro-inflammatory cytokines, which is "correlated with the production of anti-inflammatory/immunosuppressive cytokines such as TGF-beta and IL-10", they provide no data to show that their rice-derived rhLF actually has this effect. The data that are available with rhLF (Agennix's talactoferrin) in both animals[1] and humans[2] actually show the opposite (i.e., that orally administered rhLF leads to up-regulation of the pro-inflammatory cytokine IL-18).

The plant-based glycosylation is another point of concern with regard to the use of rice-produced rhLF. I particularly have difficulty accepting the argument by Ventria that "the differences in the glycosylation between native human lactoferrin and rice-produced rhLF should be of no immunological concern because it is the presence or absence of glycans that matter rather than structural differences in glycosylation."

- In my view, the fact that many self-reactive T cells recognize glycoproteins (as acknowledged by Ventria), raises the concern that rice-derived rhLF may break tolerance.

- Published data shows that the recognition of carbohydrates influences the development of arthritis, "since carbohydrate-depleted type II collagen (CII) induced arthritis with a later onset, lower incidence, and milder symptoms compared to arthritis induced with glycosylated CII." [3]

- Many potential targets for self-reactive T cells are in fact glycoproteins and can be associated with autoimmune disease. As summarized by Anderton [4], post-translational modifications (PTMs) of mammalian proteins, including glycosylation "can allow immune recognition of neo-self epitopes. These effects can vary from overt increase in affinity of MHC or T-cell receptor binding, to more subtle effects on the activity of proteolytic enzymes involved in antigen processing." Further, it is well known that changes in glycosylation can influence antigenicity and may affect several parameters of the immune response including antigen processing and the type of immune response elicited.

- Backlund et al [5] specifically evaluated the role played by glycosylation in immune tolerance. The investigators used a human DR4-transgenic mouse model to determine the impact of glycosylation on the induction of CII specific T cell tolerance. An important conclusion of the study is that "in DR4-transgenic mice expressing huCII, and most likely also in humans, T cells recognizing the nonglycosylated 263–270 epitope are strongly tolerized, or even deleted, whereas T cells specific for the different glycosylated peptides persist." Beyond looking at the presence or absence of glycosylation, the investigators also evaluated the effect of relatively minor changes in the carbohydrate structure. In addition to nonglycosylated peptides and peptides glycosylated with galactose, they synthesized peptides glycosylated with 2-deoxy, 3-deoxy or 4-deoxy galactose. Each entity yielded a distinct response profile, demonstrating that even minor changes in the carbohydrate structure have a substantial impact on immune recognition. As pointed out by the investigators, "the relevance of the findings made in mice expressing huCII and DR4 is evident because 30% of the investigated RA patients exhibited a predominant response to the glycosylated forms of the CII263–270 epitope. The investigators go on to conclude that their finding "show that the physiological post-translational modification of variable carbohydrate attachment converts the immunodominant naked self-peptide, which is tolerogenic, into several cryptic self-determinants that remain immunogenic."

Given these facts, I find it difficult to imagine that rhLF produced in rice would be suitable for a GRAS designation that could result in the availability of unlimited amounts of rhLF to individuals without medical supervision. I hope these comments are helpful. Please do not hesitate to contact me if you have additional questions.

Kind Regards,

Margalit Mokyr

Margalit B. Mokyr, Ph.D.
Professor

Cc: Robert Merker, Ph.D. (HFS-255)
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Re: Safety Concerns Raised by Recombinant Human Lactoferrin from Rice
(GRN No. 000162 Submitted by Ventria Bioscience)

Dear Dr. Tarantino:

I am writing to provide some additional brief comments pertaining to the September 8, 2006 scientific assessment of issues concerning rhLF produced in rice.

Based on the ethics of newborn and infant care and the established principals governing human experimentation, I am a signatory to this communication. I strongly agree with the conclusions that were presented and, based on my many years of medical experience, do not believe that rhLF is a compound suitable for unregulated human consumption.

In addition to the facts presented in our submission, additional rationale needs to be articulated. First, three studies in foreign countries say probiotics can prevent neonatal necrotizing enterocolitis. Probiotics have been regarded as having a relatively benign safety profile (as Ventria asserts for their product), yet no U.S. probiotic product has so far been approved by the FDA for use in neonates. In my collaborative work at UC Davis, we are completing the first U.S. trial of probiotics in neonates. The study required institutional review and an independent data monitoring committee was mandated by the IRB. I have been told that the NICHD Neonatal Network and the Vermont Oxford Network will be required by the FDA to obtain an IND before initiating any clinical trials of probiotics in neonates. Probiotics may be considered an enteral nutritional agent in adults, but human experimentation on neonates necessitates caution in treating this vulnerable patient population. Similarly, before rhLF of any variety is used on neonates or other vulnerable populations, ethics demand the conduct of randomized, controlled clinical trials of adequate duration to demonstrate its safety with long term use. In another example, bovine surfactant containing surface-active proteins required years of clinical trials to demonstrate efficacy and safety before market approval was granted. This is an ethical issue that should receive strong consideration.

The second point I would like to mention concerns the use of rhLF as a biologic agent. Lactoferrin in human milk has no known role in infant nutrition (i.e., it is NOT essential for weight gain or as a growth factor). Rather, the role of lactoferrin is to serve as a modulator of innate host defense. It is therefore hard to understand why rhLF would be regarded as a food additive rather than a drug, since its core function is that of an immumodulatory agent and a natural protein antibiotic.

Thank you for your consideration of my comments. Please do not hesitate to contact me if I may be of further assistance. My cell phone number is: 217-725-6174.

Sincerely yours,

Michael P. Sherman

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May 20, 2005

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AgenNX

Re: **Ventria GRAS Notice No. 162: Use of Recombinant Human Lactoferrin In Food and Dietary Supplements**

Dear Drs. Tarantino, Schneeman, and Martin,

With respect to the petition for GRAS listing of recombinant human lactoferrin (rhLF) by Ventria BioScience (GRAS Notice #162), we respectfully submit the following public documents for consideration:

"Consumers Union's Comments on USDA Animal Plant Health Inspection Service (APHIS) Environmental Assessment for Field Test of Permit of Ventria Bioscience rice genetically engineered to express human lactoferrin USDA/APHIS Docket No. 05-006-1"
Consumers Union – March 2005

Eight Greenway Plaza Suite 910 Houston Texas 77046 Telephone 713/552-1091 Facsimile 73/552-0795

"Comments on APHIS Environmental Assessment for Permit Application No. 04-302-01r for Outdoor Cultivation of Rice Expressing a Novel, Recombinant Human Lactoferrin Submitted to USDA's Animal and Plant Health Inspection Service Docket No. 05-006-1"
Friends of the Earth - March 25, 2005
(Particularly pages 11-12; 18-22; 34-35)

"Comments on Two Environmental Assessments on Permit Application Number 04-302-01r: Ventria Rice Expressing Lactoferrin (Docket 05-006-1), and Permit Application Number 04-309-01r: Ventria Rice Expressing Lysozyme (Docket 05-007-1)"
The Center for Food Safety - March 24, 2005
(Particularly pages 14-16)

These public documents were submitted by respected consumer advocacy groups in response to Ventria's requests for approval to grow GMO Pharma rice containing recombinant human lactoferrin. The documents point out that recombinant human lactoferrin has not been shown to be safe for general human consumption, and in fact, depending on the full recombinant sequence (which Ventria has never determined or disclosed) and the specific glycosylation, it could be hazardous. There clearly is not a consensus within the scientific community that recombinant human lactoferrin is safe for its intended use and thus it fails to meet the requirements for being considered GRAS.

Additionally, as discussed in the previous submission in this matter by our legal counsel (Peter Barton Hutt and Ruth Miller of Covington & Burling), recombinant human lactoferrin is, and has been, in active clinical development as an investigational new drug regulated by the FDA (CDER), with open INDs in the U.S. since 1996. Granting the present request for GRAS listing would directly contradict recombinant human lactoferrin's existing regulatory status with the FDA as an investigational new drug.

We believe the information submitted currently, together with our previous submission, clearly demonstrates that **Ventria's petition for GRAS listing of recombinant human lactoferrin should be DENIED.**

Please do not hesitate to contact us at the address or phone number below if we can provide any additional information that would be helpful.

Sincerely,

Rick Barsky

Rick Barsky
Chief Executive Officer

Attachments

Consumers Union's Comments on
USDA Animal Plant Health Inspection Service (APHIS)
Environmental Assessment for Field Test of Permit of
Ventria Bioscience rice genetically engineered to express human lactoferrin
USDA/APHIS Docket No. 05-006-1

Summary

Consumers Union* appreciates the opportunity to comment on the USDA/APHIS Environmental Assessment (EA) for Ventria's plant-made pharmaceutical (PMP) rice variety that has been genetically engineered to express human lactoferrin. We restrict our comments to issues associated with the human (and animal) health. For the reasons laid out below, we believe that the EA is inadequate and contains internal inconsistencies. Consequently, we urge USDA/APHIS to deny Ventria's permit application and to not issue any further permits for field trial of any of Ventria's PMP rice varieties before a full regulatory review has been completed by FDA and EPA on the human health and ecological implications, respectively, of this PMP rice variety. In our view, USDA should not grant permits for any outdoor trials of PMPs. However, it certainly should not do so before FDA has adequately addressed any safety issues associated with the PMP. We also believe that USDA/APHIS should not regard this EA as final until the potential impacts on non-target species—particularly birds (especially waterfowl) and rodents—have been investigated in more scientific detail.

We have found a number of serious deficiencies and potential inconsistencies in Ventria's EA vis-à-vis the potential human health impact of their PMP rice variety expressing human lactoferrin. Many of the deficiencies relate to issues associated with allergenicity. Allergenicity is one of the key potential human health impacts associated with genetically engineered proteins. It is important to assess any risk that recombinant human lactoferrin (rhLf), a protein normally found in milk (among other body fluids), but which is being introduced here into rice, might pose, especially to people with milk allergies. Although there is no

single test for allergenicity, there has been global agreement on the types of tests that should be done (FAO/WHO 2001). In particular, characteristics of allergens include: that they have amino acid sequence similarity to known allergens, they are glycoproteins, they have resistance to digestion, and display heat stability.

Ventria FDA Submission Contradicts EA on Sequence Homology

The EA states that Ventria's studies conclude that "no [amino acid] sequence homology of the recombinant lactoferrin to known toxicants, allergens, or proteins likely to harm non-target organisms. . . . The SWISS-PROT AND TrEMBL databases were utilized and no amino acid sequence homology was found between lactoferrin and known allergens." italics added. This statement appears to directly contradicts statements Ventria made in their November 24, 2003 submission to FDA: "A search against a database of known allergens, identified in the SWISS-PROT and TrEMBL databases, also using the techniques described by Gendel, found 52% amino acid sequence homology between human lactoferrin and chick ovotransferrin (conalbumin), which is a known allergen" italics added (Bethell, 2003: 46). Ventria then seeks to dismiss this finding by arguing that human lactoferrin can't be an allergen because i) it's found in human breast milk and ii) it has never been identified as an allergen. But the fact remains that Ventria did find sequence similarity between lactoferrin and a known human allergen.

We are very concerned that Ventria's conclusions in these two documents appear to directly contradict each other. We believe the EA should be revised to incorporate the information submitted to FDA and that the implications of this information in terms of allergy risk should be fully addressed rather than dismissed as Ventria does. We are especially concerned that risks to people with milk allergies be addressed. A prominent French allergy specialist, Dr. Jean-Michel Wal, found that 41 out of 92 milk-allergic patients had detectable IgE antibodies to bovine lactoferrin and concluded that bovine lactoferrin may be an

important allergen (Wal, 1998). Bovine and human lactoferrin share 68% amino acid identity (Groenink et al., 1999).

Both Codex Alimentarius ((CAG/GL 45-2003) and a global expert meeting on allergenicity (FAO/WHO, 2001) have proposed that any global sequence homology between a transgenic protein and known allergen that exceeds 35% would be considered significant and should trigger further research. The sequence similarity between rhLf and the egg allergen conalbumin and bovine lactoferrin is 52% and 68%, respectively. Both figures greatly exceed the 35% threshold suggest by both Codex Alimentarius and FAO/WHO. These data clearly show that rhLf has significant sequence homology with two known allergens. These findings should clearly trigger further research into the potential allergenicity of rhLf.

In order to assess sequence similarity to known allergens, Ventria must be required to provide the complete amino acid sequence information for their rhLf. The exact amino acid sequence of the plant-derived lactoferrin has not been determined. When comparing the physical and chemical properties of recombinant human lactoferrin (rhLf) and purified human lactoferrin (hLf), the EA notes that "N-terminal sequence were found to be identical"; on this basis APHIS concludes that amino acid sequence of rhLf and hLf are identical. However, the EPA's Scientific Advisory Panel has specifically stated that this approach is inadequate and has recommended full-length amino acid sequencing of plant-produced recombinant proteins, noting that one or two point mutations can modify allergenic property: "sequence similarity of full length amino acid sequence (highly undesirable is the sequence analysis of 10-15 N and/or C-terminal amino acids and up to three short internal protein sequences). For example, two isoforms -lactoglobulin i.e. genetic variants which differed only by two□of point mutations on residues 64 and 118 (D and V versus G and A) showed modified allergic properties [Wall, 1998]" italics added (SAP, 2000, pg. 14). Furthermore, the process of genetic engineering is not exact, so that during

the insertion process, small point mutations may occur in the inserted DNA that could lead to amino acid substitutions in the plant-produced rhLf.

Further, under molecular characterization, the EA states that Ventria's data demonstrate "that there are approximately 6 copies of the lactoferrin coding sequence integrated into the rice genome." However, in the submission to FDA, Ventria states that "it is estimated there are 8 copies of the complete 3156 bp chimeric lactoferrin expression cassette" (Bethell, 2003: 20). This apparent discrepancy between data submitted to USDA/APHIS and data submitted to FDA should be addressed and resolved before the EA is considered final.

EA Contradicts Itself on Resistance to Digestion

The EA suggests that rhLf is rapidly digested and so wouldn't be a problem (one of the characteristics of known allergens is stability to digestion). In the in vitro pepsin digestion experiment, "Ventria's rice-derived lactoferrin (rhLf) is equivalent to native human lactoferrin (hLf) which is rapidly degraded (<30 sec) in gastric fluid." This rapid digestion (<30 seconds) clearly suggests that rhLf doesn't survive digestion. However, we note that Ventria proposes to extract rhLf from the rice and use it "as supplements in yogurts, meal replacement and performance beverages, bars (for example granola bars), and in nutritional supplement drinks." If rhLf is degraded as quickly as Ventria suggests, then it wouldn't be orally active. Yet, since rhLf is supposed to have beneficial (antimicrobial) activity, then it would have to be orally active. If it isn't orally active, then there would be no real benefit to putting it into the suggested products.

In addition, Ventria refers to a study where chicks fed the transgenic rice had improved health and growth rates compared to controls. This study clearly shows that the rhLF (and recombinant human lysozyme) in the rice was orally active, suggesting that it does survive digestion. In addition, this chick study also noted

that previous studies found that lysozyme and lactoferrin "are highly resistant to hydrolysis by acids and proteases and to digestion in the gastrointestinal tract" italics added (Humphrey et al., 2002: 1214). Indeed, one of the cited studies is titled, "Functional fragments of ingested lactoferrin are resistant to proteolytic degradation in the gastrointestinal tract of adult rats" (Kuwata et al., 2001). Finally, a study done by Dr. Bo Lonnerdal using Ventria's rhLf also demonstrated that that rhLf is resistant to digestion in an in vitro digestive system (Lonnerdal, 2002). Dr. Lonnerdal's system consisted of exposing the recombinant human milk proteins to low pH and pepsin for 30 minutes at 37 C, then adjusting the pH to pH 7, adding pancreatin (mixture of pancreatic enzymes), and then incubating for 30 to 60 minutes. Dr. Lonnerdal found that for "all three proteins [recombinant versions of -1-antitrypsin] we studied, activities□human lactoferrin, lysozyme and remained after treatment" (Lonnerdal, 2002: 220S). So, the data do appear to show that rhLf does survive gastric digestion.

We are very concerned that this EA contains these internal inconsistencies and believe they should be addressed before this document is considered final. In addition if, as appears to be the case, rhLf is orally active and effects growth of birds, this has important environmental implications. What are the consequences if this rice is consumed by wildlife? What might be the effect on ecological relationships? Could there be dramatic population increases in pest birds or mammals, such as Canada geese and starlings or mice, due to their increased health? These issues must be explored.

EA Contradicted by Omitted Studies on Heat Stability

The EA also suggests that rhLf is not heat stable, noting that after using a commercial rice cooker for 20 minutes, "lactoferrin protein could no longer be detected." However, a heat stability study done by Dr. Lonnerdal, published in the Journal of the American College of Nutrition, found rhLf to be heat stable. Dr. Lonnerdal did the heat stability studies as part of research into the use of rhLf in

infant formula and baby foods. As Dr. Lonnerdal noted: "If recombinant human milk proteins are to be added to infant formula or baby foods, some degree of processing may be involved. We therefore exposed the -1-antitrypsin],□recombinant proteins [human lactoferrin, lysozyme and both in pure form in solution and as added to infant formula, to various heat treatments, ranging from 78-100 C for 8 seconds up to 30 minutes. Except for the most severe treatment, 100 C for 5 minutes, which partially inactivated both recombinant and native human milk proteins, these proteins maintained activities similar to those of the native proteins" (Lonnerdal, 2002: 220S). Thus, rhLf does appear to be heat stable. The implications of this information in terms of allergy risk should be thoroughly addressed.

The EA states that the "only biochemical difference detected between the recombinant lactoferrin and the purified human lactoferrin were differences in glycosylation patterns." The EA dismisses this difference by noting that "Ventria has demonstrated that the digestibility between rhLf and hLf are similar, showing that the stability of the protein has not been affected by glycosylation." Since most known human allergens are glycoproteins, the fact that rhLf is glycosylated differently than the native hLf is potentially very significant. The different glycosylation patterns between rhLF and hLf could have a direct impact on the potential allergenicity. The fact that there may be no real change in digestibility is not comforting because there is clear evidence that hLf is resistant to digestion. The implications of theses findings in terms of allergy risk should be fully discussed and addressed.

Conclusions on Allergenicity

In sum, in terms of allergenicity, rhLf does have a number of traits—sequence similarity to known allergens, heat stability, resistance to digestion, being a glycoprotein—in common with known allergens. The fact that bovine lactoferrin may be an allergen is also of concern. Although Ventria, in their submission to

FDA, argues that rhLf can't be an allergen because it's found in breast milk, their reasoning is spurious. By this reasoning, any human-derived drug couldn't cause allergic reactions because the drug is normally found in the human body. However, it is well known that protein drugs derived from humans and produced via genetic engineering, can provoke an immune system response to the recombinant human protein and sometimes to the native human version. Thus, some patients receiving recombinant blood clotting Factor VIII and the multiple sclerosis drug beta-interferon develop antibodies to the recombinant proteins, which reduces the drug's potency (Freese et al., 2003). In another case, Amgen's recombinant megakaryocyte growth and development factor (MGDF) was discontinued in clinical trials after some patients developed antibodies to both the recombinant MGDF as well as the natural version of MGDF, which resulted in bleeding. Such immune system reactions to recombinant human proteins were unexpected as the recombinant and human versions of the proteins were identical in amino acid sequence and glycosylation patterns. Even though the scientists couldn't detect differences between the recombinant and human versions of the proteins, the immune systems of the affected patients were able to distinguish between them. If these human protein drugs, produced using tightly-controlled fermentation using mammalian cell culture, could result in such unexpected immune system effects, why should we think that something similar could not happen with rice-produced rhLf compared to hLf?

Finally, since rhLf does have a number of traits—sequence similarity to known allergens, heat stability, resistance to digestion, being a glycoprotein—in common with known allergens and since rhLf has a different glycosylation pattern than hLf, there still remains a question about the potential allergenicity of rhLf. Perhaps that is why noted food allergy expert Dr. Steven Taylor of the University of Nebraska stated that FDA's regulations "will have to be rethought before rice-grown lactoferrin, and other human proteins made by genetically modified organisms, can be approved for production" (Pearson, 2002:).

If rhLf is a potential allergen, then the issue of possible mixing with or contamination of conventional food rice becomes a serious concern. These possibilities need to be much more fully evaluated in the EA.

Other Potential Human Health Effects

Recombinant human lactoferrin could have other adverse health effects on humans besides potential allergenicity. Lactoferrin is found in numerous body secretions and has antibacterial, antimycotic, antineoplastic and anti-inflammatory activities. Lactoferrin is part of the body's "iron withholding defense system," which serves to find and bind to toxic quantities of iron. Since many microbes need iron, lactoferrin serves to starve those microbes of the needed iron. Work has shown that lactoferrin can suppress bacteria such as Listeria monocytogenes. Other work shows that lactoferrin and lysozyme act synergistically to protect against various microbes, including E. coli, Salmonella typhimurium and Vibrio cholerae; individually there's no negative effect on the bacteria (Ellison and Giehl, 1991).

While rhLf's iron-binding properties make it a good defense against various microbes, there are microbes that can feed off the iron in lactoferrin and thereby possibly become more of a problem. Microbes such as the Trichomonas protozoa (Weinberg, 1999), Helicobacter pylori (Dhaenens et al., 1997) as well as members of the bacterial family Neissariaceae (which contains numerous venereal disease bacteria) (Vogel et al., 1997) can obtain iron from human lactoferrin. Since H. pylori is the major etiologic agent of chronic gastritis and a component of the etiology of gastric ulcers and carcinomas, supplying rhLf to people with H. pylori infections could make the infections worse rather than better.

Another potential adverse effect of rhLf is that is that it could induce an antibody response in some people. Antibodies to natural lactoferrin have been found in patients with autoimmune diseases such as systemic lupus erythematosis,

rheumatoid arthritis and primary scerosing cholangitis (Skogh and Peen, 1993; Afeltra et al., 1996). Since rice-produced rhLf is glycosylated differently than native hLf, its allergic potential could be increased. The finding of antibody responses to many of the human protein drugs produced via genetic engineering shows that an immune response to a recombinant version of a human protein can happen.

As a result of the potentials for stimulating certain pathogens (by being a source of iron) and for provoking an allergic response, some scientists have advised a bit of caution in promoting the use of rhLf for disease reduction. Dr. Eugene Weinberg, of Indiana University, wrote a review article on the potential uses of rhLf in treating various medical conditions in which he cautioned, "an adverse response to the protein might occur if it were to stimulate antibody production or if it were to provide iron to the invading pathogen. . . . Precaution is needed; however, to avoid antigenic sensitization as well as introduction of the protein to tissues that may be infected with specific protozoa or bacteria that utilize Lf in their acquisition of host iron" (Weinberg, 2001).

Conclusion on Potential Human Health Effects

In sum, as we have discussed, there are serious inadequacies in this EA with respect to potential human health effect. These inadequacies must be fully addressed in any EA prior to granting a permit.

In our view, USDA should not grant permits for any outdoor trials of PMPs. However, it certainly should not do so before FDA has adequately addressed any safety issues associated with the PMP. In this regard, we note that Ventria submitted a "Food and Feed Assessment Summary" to FDA on November 23, 2003 as part of the voluntary consultation process for genetically engineered plants. To date, FDA has not issued a letter saying that they have no further concern, suggesting that FDA has further questions on this compound.

RhLF May Have adverse effects on non-target birds and mammals

The finding that certain human pathogenic bacteria and protozoa can be increased by the presence of rhLf also raises the possibility that other bacteria and protozoa that are pathogenic for various animal species, particularly birds, could also be increased by the presence of rhLf. USDA/APHIS argues that there will be no adverse impact on bird species that eat the rice, based on the single chick feeding study that found beneficial effects. That study did not involve chicks that were infected with any pathogens that may be able to obtain iron from rhLf. Thus, one cannot conclude from this single chick study that there would be no adverse effects on birds. USDA/APHIS should determine if there are any pathogenic microbes that infect birds that have the ability to obtain iron from lactoferrin and determine what would happen if they consumed Ventria's rhLf-producing rice. Since the rhLF levels are very high in Ventria's PMP rice, any iron-scavenging pathogens could significantly increase their numbers. Since rodents and other mammals could also consume the rice before or after harvest (rice spilled during harvesting), they could be adversely affected as well. It's also possible that the potential protective effects of rhLf could be greater than the negative effects on birds and rodents. In either case, the possibility exists that there could be a disruption in the ecosystem as a result of consumption of this rice by birds or rodents. Thus, the EA is inadequate on this point. We therefore believe that USDA/APHIS should not regard this EA as final until the potential impacts on non-target species—particularly birds (especially waterfowl) and rodents—have been investigated in more scientific detail.

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Notes:

* Consumers Union is a nonprofit membership organization chartered in 1936 under the laws of the State of New York to provide consumers with information,

education and counsel about goods, services, health, and personal finances; and to initiate and cooperate with individual and group efforts to maintain and enhance the quality of life for consumers. Consumers Union's income is derived solely from the sale of Consumer Reports, its other publications and from noncommercial contributions, grants and fees. In addition to reports on Consumers Union's own product testing, Consumer Reports, with approximately 4.5 million paid circulation, regularly carries articles on health, product safety, marketplace economics and legislative, judicial and regulatory actions which affect consumer welfare. Consumers Union's publications carry no advertising and receive no commercial support.

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March, 24, 2005

Docket No. 05-006-1 and 05-007-1 (submitted separately to each, in quadruplicate)
Regulatory Analysis and Development, PPD
APHIS Station 3C71
4700 River Rd., Unit 118
Riverdale, MD 20737-1238

Re: Comments on Two Environmental Assessments on Permit Application Number 04-302-01r: Ventria Rice Expressing Lactoferrin (Docket 05-006-1), and Permit Application Number 04-309-01r: Ventria Rice Expressing Lysozyme (Docket 05-007-1)

Dear Sir/Madam:

The Center for Food Safety (CFS) appreciates the opportunity to comment on the above-referenced APHIS Environmental Assessments (EAs) on Ventria Bioscience's proposal to grow genetically engineered (GE) rice that expresses human lactoferrin and lysozyme, both at the same location in Missouri.

CFS believes that these field tests present potentially significant environmental impacts and associated human health risks that have not been adequately addressed in the two EAs. In general, we agree with the recent National Academy of Sciences report that concludes that, food crops are usually not a good choice for the production of pharmaceutical crops due to the difficulty of ensuring that contamination of food will not occur.[1] Similarly, an extensive review by scientists with expertise in relevant disciplines also concludes that the use of food crops to produce pharmaceuticals is ill advised.[2] The authors of that report conclude that although it would be hypothetically possible to ensure that contamination would not occur, in practice, due to the nature of commodity crop production, the prevention of contamination cannot be

guaranteed in today's agricultural environment. This is especially true when the pharm crop is

not geographically isolated from the food crop.

NATIONAL ENVIRONMENTAL POLICY ACT CONCERNS WITH BOTH EA'S

It is remarkable that both EAs address exactly the same applicant, same affected environment, same crop, and same classes of foreseeable impacts. Yet, neither EA **even mentions** the existence of the other proposed field test. Neither EA addresses the cumulative impacts of the two projects at the same site or the possibility of any synergistic effects between the two proposals. Thus, the "Cumulative Environmental Effects" sections of each are facially inadequate. This defect underscores the suitability of submitting this joint comment on both EAs, to urge APHIS to consider the impacts of the two proposals cumulatively.

Both EAs are inadequate in their descriptions of the "Need" for the proposals, that is, the need that Ventria seeks to meet with these field tests. The existing "Purpose" description for both EAs is incorrectly placed in "VII. Description of the Field Test/Affected Environment". It should be moved to the existing "II. Purpose and Need" section where it belongs, and should be expanded on as it is now too sparse to tell the reader what Ventria's aims are. Further, the Proposed Action that requires analysis here is not your agency granting a permit, as the EAs put forth, rather the action is Ventria undertaking the field tests. This conceptual confusion weakens the analysis in the EAs throughout.

Both EAs fail to adequately describe the features of the Affected Environment for the proposal. A fundamental problem is the excessive claims of Confidential Business Information (CBI) by Ventria and the allowance of these claims by APHIS. Perhaps most important are the withholding of the actual location and acreage of the proposals (p. 4 of both EAs). Knowing the location and size of the field plots is vital for determining where, how, and to whom potential unintended exposures could occur, which are key components in determining risk to the public and the environment.

Further, field test locations are **not** CBI under Federal law. This is the ruling by Federal judges in Hawaii, in a lawsuit involving GE pharmaceutical crop field tests, in the only judicial opinions to date that have considered the question. The attached two Orders, in the case of CFS et al. v. Veneman at al., in which the defendants are the Secretary of Agriculture, the Under Secretary for Marketing and Regulatory Programs, the Administrator of APHIS, and the Deputy Administrator in charge of BRS, bind your agency. (Order of U.S. Magistrate Judge Barry M. Kurren dated June 29, 2004, Denying Defendants' Consolidated Motions for a Protective Order, affirmed by Order of U.S. District Court Judge David A. Ezra dated Aug. 3, 2004, Affirming Magistrate Judge's Order for Discovery etc., U.S. District Court, District of Hawaii, civil case no. 03-00621.) At page 3 of Magistrate Judge Kurren's Order, he unambiguously states: "Field test site locations do not constitute confidential commercial or trade secret information." Those orders directed the USDA defendants to provide the claimed-CBI locations in Hawaii to CFS and others, which they have since done.

To bring your policies into alignment with the law, we urge you to now end your past practice of treating locations as CBI, not just for these two EAs, but for all public documents related to all GE crop field tests. Until then our comments are provisional because any final conclusions

about safety or lack thereof depend on the location and size of the proposed planting. Ventria should not be allowed to grow commercial quantities of GE pharmaceutical compounds for a multi-year span - as it asserts it intends to do - on hundreds or even thousands of acres under APHIS's field test regime, without revealing where. The EAs are inadequate on that basis alone.

Although the permit applications evaluated in these EAs are for a single year, Ventria is proposing to grow rice containing lactoferrin and lysozyme in southeastern Missouri for an indefinite period of time as it seeks to commercialize these products. APHIS cannot reasonably rely solely on the current EAs in assessing the risks from future field tests of Ventria's pharmaceutical rice, as is strongly suggested by statements in the EAs, despite the fact that food rice production has been dramatically increasing in the county where the field tests are proposed for.[a] The 8-9 fold increase in production of food rice over an 8 year period in the area is not acknowledged or recognized in the EAs, but could seriously impact the ability to prevent contamination, especially if the trend continues.

SCIENTIFIC CONCERNS WITH THE LACTOFERRIN EA (DOCKET 05-006-1)

In sum, the EA for lactoferrin rice is inadequate for the following reasons:

- APHIS sets an inadequate 1/4 mile isolation distance to separate Ventria rice from food rice or the serious weedy relative, red rice. APHIS has underestimated gene flow from rice in the past (based on recently published research) and has seriously underestimated the ability of genetically engineered creeping bentgrass to contaminate surrounding wild relatives.[3] These and other cases are symptomatic of inadequate data on the ability for

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[a] APHIS remarks on page 5 of both EAs: "This EA is prepared because the applicant intends to have plantings of this engineered plant in Scott County, Missouri, for the next several years. The potential for cumulative impacts of repeated plantings in the same area raises new issues that ***this*** EA addresses. Future plantings are anticipated to increase in size and will be required to meet all the performance and mitigation measures described in ***this*** EA, standard and supplemental permit conditions, and the permit application." (emphasis added). Therefore, it appears that APHIS intends that the current EAs will be adequate to address both current *and future* cumulative risks. This would contravene NEPA, which requires that an agency's environmental assessments be based on just the project proposal that is before it at the time. Thus, APHIS should issue a revised EA that corrects this suggestion of prejudgment of expected future permit proposals by Ventria.

gene flow from crops to occur. The 1/4 mile isolation distance accepted by APHIS is unlikely to ensure that gene flow will not occur.

- APHIS allows farm equipment used with Ventria's rice to be used with food rice after cleaning, despite the inability to ensure that such cleaning can remove all of Ventria's rice, which could then be transferred to food rice or contaminate fields containing red rice. A recent report by experts on farm practices confirms that complete cleaning cannot be ensured.[4] Also, there is no requirement to clean farm machinery that was previously used on conventional rice farms prior to use on Ventria's rice, which could allow the contamination of Ventria fields by weedy red rice from a conventional farm. Because of its long seed dormancy, once in Ventria's fields, red rice could be very difficult to eradicate and would likely hybridize with Ventria's rice.

- Isolation distance must not be considered alone unless prevention of gene flow can be ensured. However, APHIS admits that minimal amounts of gene flow may occur with Ventria's rice. In conjunction with the amount of gene flow, such as by cross pollination, the ability of the transgene to confer a competitive advantage to wild red rice must be carefully considered because enhanced competitive ability can facilitate permanent escape and spread of transgenes even when very low levels of gene flow occur. APHIS does not consider the substantial possibility that the lactoferrin gene could confer a competitive advantage to wild red rice by reducing disease of red rice grain, despite the fact that lactoferrin has been used in transgenic crops in previous field tests for the expressed purpose of reducing disease in those plants.

- APHIS does not consider important recent data, and thereby may seriously underestimate the possibility of horizontal gene transfer of the lactoferrin gene to bacteria, and possible risk should that occur.

- APHIS as an agency is not qualified to determine the potential human safety risks from lactoferrin rice; further, it has not adequately evaluated those risks in the EA.[b] APHIS has not considered the possible immunological (such as allergy) implications of differences between lactoferrin produced in humans, the source of the gene, and lactoferrin produced in rice, which is chemically different than the human version.

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[b] APHIS has consulted with a representative from FDA to help in its human safety determination, however, FDA has not completed its own human safety assessment, so this assistance is unlikely to be adequate.

- APHIS apparently accepts evidence that lactoferrin is degraded in the stomach, which would reduce its risk, contrary to evidence presented by Ventria that intact lactoferrin can be found in infant stool.

- APHIS accepts that lactoferrin is denatured by cooking despite questionable testing methods. More importantly, denaturation does not assure that a protein will not be an allergen, although APHIS is apparently reassured by these data.

Environmental Safety of Human-Derived Lactoferrin Produced in Rice

Gene Flow from Lactoferrin Rice to the Wild Relative, Red Rice

A critical issue for the environmental safety of pharmaceutical crops is gene flow to wild crop relatives. As the EA notes, there appears to be one wild sexually compatible relative of cultivated rice in the U.S., annual red rice, *Oryza sativa* ssp *sativa*. Cultivated rice can sometimes become weedy as well, and can grow as a volunteer feral plant in rice growing areas. Red rice is a serious weed of rice, and is reported to be found in Missouri.[5] In fact, some sources consider it to be the most serious rice weed in Missouri.[6] Red rice often readily hybridizes with, and produces fertile progeny with, cultivated rice that are often more vigorous than either parent, which could enhance survival after gene flow at low frequencies.[7] Furthermore, APHIS likely underestimates the potential for gene flow because it considers rice to be primarily self-pollinating. Although true, this assessment ignores data by Langevin and colleagues who found hybridization of red rice with a cultivar of commercial rice at 52%, which indicates the potential of a high rate of outcrossing under at least some circumstances.[8]

If the lactoferrin rice (hereafter "rL") gene contaminates red rice, it could spread well beyond Ventria's field test sites. Furthermore, because red rice seed has extended dormancy, and can survive in the soil for at least 6 or 7 years, once red rice is contaminated, it would be very difficult to eradicate or prevent further spread.

The EA discloses several permit conditions intended to reduce gene flow to either red rice or cultivated rice. The primary means of reducing gene flow is to require a separation distance of at least 1/4 mile between the Ventria field test and either cultivated rice or red rice. Ventria further claims that there have been no rice fields within 10 miles of the field test site. This estimated

separation is based on a personal communication from a University of Missouri extension specialist, with no indication of how this estimation was derived. As such, it should not be given much weight. In addition, as noted in the EA, it cannot be ensured that rice will not be grown closer to the field test site, so the 1/4 mile requirement must be the standard used for assessing possible gene flow. Furthermore, as noted in the EA, Ventria intends to expand its acreage over time, and to grow in Mississippi and Cape Girardeau counties as well as the current Scott County (EA, page 44, TES Worksheet). Northwest Missouri State University is collaborating with Ventria and its president, Dean Hubbard, has stated that eventually Ventria expects to increase cultivation of its pharmaceutical crops to 25,000 acres.[9] The latter acreage is 39 square miles, which clearly could make ensuring separation distances of greater than 1/4 mile improbable in these counties.

In addition, Ventria claims that the counties discussed in the field test application grow a total of 500 acres of rice (EA page 44, TES worksheet), all in Scott county. To the contrary, recent preliminary state agricultural data for 2003 for Scott county indicate 1200 acres of rice (data for Scott county alone was not previously recorded, but has been since 2003 due to increasing rice acreage). For the three other counties of Cape Girardeau, Mississippi, and Bolinger, 1400 acres were grown in 2003 (individual county data not available). Therefore, the three counties where Ventria plans on growing rL rice, plus Bolinger, grew about 2,600 acres of rice in 2003, compared to about 300 acres in 1996, or an increase of almost 9 fold in 8 years.[10] [11] In addition, surrounding counties grow large amounts of rice, for example, Stoddard County grows about 58,000 acres. Taken together, these data indicate a trend of increasing food rice cultivation in these counties, which may encroach on Ventria's rice significantly if it continues, and already questions Ventria's isolation assumptions.

On page 17 of the EA, APHIS claims that because rice pollen survives for only a few minutes (5 to 20 minutes is often cited), the 1/4 mile separation from food rice or red rice would reduce gene flow to "*de minimus*" levels. The EA does not define what is meant, in practice, by "*de minimus.*" Unfortunately, APHIS assurances about adequate confinement distance have been repeatedly proven wrong, primarily because data on low levels of gene flow that could be important when considering pharmaceutical rice and pollination of wild relatives have generally not been available. For example, APHIS previously required only a 10 foot separation distance for GE rice, based on seed purity standards for conventional breeding. APHIS then increased this distance to 100 or 200 feet for pharmaceutical rice. However, recent research indicates that gene flow may occur at 110 meters, between a quarter and a third of the 1/4 mile distance required separation distance.[12]

Furthermore, the maximum distance for rice pollen dispersal estimated in this research was based on only a few measurements in calm weather, and was extrapolated from those measurements using regression analysis, and where the largest pollen source was only 72 m^2, compared to the reported 204 acres for the Ventria field tests. This method of distance estimation is unlikely to adequately account for either the leptokurtic distribution often seen in pollen flow measurements (i.e., instead of pollen dispersal diminishing in a regular manner to zero over distance, low levels of pollen can be found at very long distances from the source), nor account for weather conditions that could substantially increase pollen dispersal, for example thunder storms, high winds, or air turbulence. The authors note that their suggestion of a 110 meter isolation distance is based on "normal weather conditions." They also note that: "Undoubtedly, a detailed study is needed to find out what the influences of other weather parameters are on the distribution of rice pollen flow under normal field conditions."[13]

The approach of limited incremental increases in isolation distance compared to previous standards, as proposed by Ventria, does not address the fundamental lack of sufficient data on pollen dispersal. Because few experiments have been carried out that include a range of environmental conditions, current data cannot be considered to represent maximum distances for gene flow. The most recent example of APHIS underestimation of pollen dispersal was an experiment with GE creeping bentgrass where gene flow was found at the greatest distances measured - about 13 miles from a field test - which was miles farther than expected based on the much more limited confinement requirements that APHIS placed on that field test.[14] Similarly, although APHIS typically requires a 1/4 mile isolation distance for genetically engineered (non-pharmaceutical) canola, recent experiments show small amounts of gene flow at 3 kilometers.[15]

All of these distances should be considered preliminary, because various conditions may affect the results by affecting the rate of pollen travel and survival time. Factors such as wind speed, air turbulence, ambient temperature, cloud cover (UV light penetration), and humidity may all affect the distance over which gene flow may occur. In addition, the relative sizes of the pollen source (Ventria rice fields) and red rice stands is likewise important, because a large pollen source and relatively small number of accepting plants may increase gene flow.[16] [17] Although APHIS inappropriately does not reveal the size of the field test, press reports citing Scott Deeter of Ventria indicate that their field test applications (presumably for lactoferrin and lysozyme) are for about 200 acres.[18] It is therefore likely that the source of rL pollen will likely be much greater than the size of red rice stands.

APHIS also does not consider observations of possible pollination of rice by insects. For example a small increase in outcrossing of rice has been observed when honey bees are present.[19]

Clearly rice is primarily wind pollinated, but low levels of insect pollination need to be considered. Honey bees often forage over distances greater than 1/4 mile, sometime over several miles. Therefore, bee or other insect pollination could be important for gene flow to red rice.

Finally, data on gene flow distances typically follow a skewed leptokurtic distribution. Graphs of gene flow distances usually show a very long distribution "tail," whereby low levels of gene flow occur over very long distances without falling to zero (at least, not within the distances observed in the experiments).[20] [c] The skewed distribution means that APHIS cannot simply examine most current, limited, data and extrapolate safe isolation distances.

Another possible means of gene flow could occur from farm machinery. In particular, machines with complex internal compartments not readily accessible from the outside may harbor small amounts of seed, even when cleaned. APHIS requires machinery used on biopharm crops to be "dedicated' to that crop. However, APHIS definition of "dedicated" allows the farm equipment to be used on conventional food crops after cleaning. In the case of Ventria, machinery such as planters, and especially harvesters, may be used with food rice after they are used for rL rice after cleaning. Ventria remarks in the EA that it intended to clean its equipment according to APHIS standards prior to use with food rice. A recent review of biopharm crops by experts knowledgeable about farm equipment concluded that such equipment cannot usually be cleaned carefully enough to ensure that biopharm seed will not be co-mingled with food seed.[21] If that occurs, such seed could either end up in the food supply, or in seed used for planting future rice crops. In the latter case, rL rice would be planted unsuspectingly, and could thereby be perpetuated in the food supply, or contaminate red rice.

There is also no requirement to clean farm equipment *prior* to use on Ventria's fields. This allows the possibility for red rice to be introduced to Ventria's fields from other rice fields. Red rice growing in Ventria's fields would be cross pollinated by Ventria's rice, transferring the rL gene. And because of its very long seed dormancy period, such rL red rice could easily evade volunteer rice control protocols, and emerge years after a particular field is no longer used by Ventria. And rL red rice, once in Ventria's fields, would not only be extremely difficult or impractical to eradicate, it may eventually escape by machinery or other means.

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[c] Recent experiments by Rieger et al. show a more random distribution, but this may have occurred due to the combination of data from several sites. In any case, those data also demonstrate higher than expected gene flow at longer distances than would otherwise be expected.

Further, morphological convergence between red rice and cultivated rice has been reported in some cases. This would make the detection of red rice growing among cultivated rice more difficult.[22] [23] This could make assessment of the proximity of red rice more difficult or inaccurate, and increase the possibility that machinery, such as combines, could unwittingly harvest red rice prior to use in Ventria's fields.

Finally, APHIS does not adequately consider the movement of seed by birds, mammals, or water. As discussed in the following section, low frequency events may be sufficient to cause permanent escape of the rL gene to red rice if the fitness of the wild species is enhanced. Therefore, the evaluation by APHIS on dispersal by birds, mammals, or flooding is inadequate because it does not consider potential impact of low levels of initial gene flow. In particular, due to the relatively short required isolation distance of 1/4 mile, incidental transfer, such as rice grains temporarily lodged in the feathers of waterfowl or the fur of mammals, could occur. Similarly, the screens and grates described by APHIS to prevent transfer by water can become clogged or overflow. Although the EA claims that the nearest "large" body of water is 4 miles from the field test site, elsewhere, page 44 of the EA notes that "The nearest non-agricultural water is more than one mile from the field." Such a location may allow the growth of volunteer rice for long enough to transfer to red rice.

APHIS's unsupported claim that the lack of observed volunteer rice at the edges of rice fields is "strong evidence" that transfer of rice does not occur is not credible without any discussion about how frequently anyone looks for such volunteer rice.

In summary, several routes of possible gene flow to red rice or cultivated food rice exist that have not been adequately considered by APHIS. The assertion that gene flow would occur only at *de minimus* levels cannot be considered credible without better data, and absent consideration of the possible fitness consequences of gene flow to red rice, considered in the following section.

Failure to Evaluate the Potential Contribution of the Lactoferrin Gene to the Fitness and Weediness of Red Rice

Isolation distance must not be considered separately from the ability of the transgene(s) to survive after gene flow occurs. Low levels of gene flow can lead to the permanent escape of the transgene if it confers a selective advantage to a wild relative, in this case, red rice.[d] Put differently, a gene that confers a fitness advantage may persist and spread in a wild relative whereas a gene that is disadvantageous may not survive or spread.[24] Therefore, fitness, or the competitive advantage conferred to a wild relative by a transgene, is of fundamental importance in determining the possibility of transgene escape unless the absence of gene flow can be ensured. As has been noted, APHIS does not ensure that gene flow will not occur, but only that it may occur at *de minimus* levels. However, without properly considering the possible fitness conferred to red rice by the lactoferrin gene, APHIS cannot properly determine what a *de minimus* level of gene flow may be.

Unfortunately, APHIS does not adequately consider the ability of the lactoferrin gene to persist and spread if it escapes to its wild relative, red rice. In fact, APHIS apparently does not even consider the possibility that the lactoferrin gene may confer a selective advantage to red rice. APHIS merely states that the lactoferrin (or hygromycin) "is [not] expected to alter the susceptibility of the transgenic rice plants to disease or insect damage." (EA, page 15) This is a fundamental shortcoming of the APHIS argument for the safety of lactoferrin rice, and in combination with inadequate analysis of gene flow and the 1/4 mile isolation distance, constitutes a major failing of the APHIS risk assessment.

In particular, APHIS does not assess the possibility that the well known antimicrobial properties of lactoferrin may confer a competitive advantage to red rice. Resistance to diseases or insects are properties that the National Academy of Sciences (NAS) and others consider to potentially confer a competitive advantage to wild crop relatives, by increasing survival compared to plants that do not possess the gene.[25] The NAS stated that: "Generally, if an allele confers a fitness advantage - once introduced into a population - it is expected to increase in frequency, even if it is introduced only once."

One of the primary commercial applications of lactoferrin is as an anti-diarrheal for infants. APHIS acknowledges in the EA that lactoferrin has been noted to have antimicrobial properties. It is especially remarkable that APHIS dismisses the possibility that lactoferrin could confer disease resistance to rice or red rice, because two field tests have been approved by APHIS for wheat containing bovine lactoferrin specifically intended to mitigate fungal disease.[26] In

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[d] A fitness-neutral transgene may also survive in red rice, but at lower numbers and with less likelihood.

addition, the gene is expressed at a very high level compared to most transgenes, approximately 4-5 mg/g. This is comparable to the levels expressed in milk, where it confers antimicrobial properties. For example, by comparison, Bt Cry proteins are typically expressed at microgram/g levels, typically several hundred to several thousand fold less than lactoferrin in rice.

APHIS repeats in its EA the same absence of rigor in determining the possible contribution of a disease resistance gene to fitness that was strongly criticized by the NAS in 2002, when APHIS failed to adequately analyze the possible consequences of virus-resistance gene flow to wild squash.[27] In that case, APHIS conducted an analysis of possible impacts of gene flow to wild squash, but the NAS found the analysis inadequate. In the case of lactoferrin, not even a cursory analysis has been conducted. APHIS merely mentions that no differences in disease susceptibility of transgenic rL rice were observed in previous field tests. However, such an analysis has little value if relevant pathogens were not present, or without discussion about how rigorous the observations were. Those previous field tests also were not conducted in Missouri, which has a somewhat different spectrum of rice diseases than states in other parts of the country (California or Hawai'i), where previous rL rice field trials have occurred. It is also possible that fungicides were applied to control fungal diseases, which would have made disease observations of little value.

As discussed in the NAS report, a proper analysis should consist of determining the susceptibility of red rice to rice seed diseases, prevalence of such diseases in Missouri, susceptibility of rL rice after inoculation with the pathogen, etc. There is no evidence that any such studies were conducted.

Because APHIS provides no explanation for its apparent lack of concern about possible competitive advantages conferred to red rice by lactoferrin, we can only speculate as to the lack of adequate analysis. One reason may be that lactoferrin is said to be expressed only in the seed of rice, rather than in the entire plant, as is the case with many other transgenes. Such reasoning would be misplaced. For example, the American Phytopathological Society lists several diseases of rice kernels, which may affect fecundity.[28] And fecundity, including survival of seed, is a major fitness component. For example, recent research found that a Bt Cry protein substantially increased survival of wild sunflower seed by protection against several lepidopteran (moth) pests, allowing more progeny to be produced.[29]

Seed often contains proteins that confer resistance to insects or diseases. These proteins include trypsin inhibitors, lectins, and alpha-amylases that inhibit insects, and pathogenesis related (PR) proteins such as chitinases and beta-glucanases that inhibit diseases.30 31 32 If these proteins

did not confer a significant advantage to the plant, they would likely not be produced because of the metabolic costs involved. However, although these endogenous pest protections are effective against many insects and diseases, the ones produced in rice seeds are not effective, by definition, against the pathogens and insects that harm rice. Therefore, it is clear that an antimicrobial protein like lactoferrin, that may confer resistance to diseases that are not controlled by endogenous antimicrobial substances in the rice grain, may confer a substantial competitive, selective, advantage to red rice.

Finally, the likelihood that gene flow will occur from continuing field tests or commercial production is greater than from a single field test. Again, Ventria has stated its intention of continuing these field tests indefinitely and expanding the acreage dramatically. Under such circumstances, contamination of food rice and red rice becomes more likely.

Horizontal Gene Transfer to Soil Bacteria from Lactoferrin Rice

APHIS does not consider horizontal gene transfer (HGT) to soil microorganisms to be a significant risk. APHIS does not evaluate important research or fitness concerns when evaluating the possibility of HGT. APHIS cites experimental data indicating that HGT occurs at exceedingly low frequencies, often undetectable, and sometimes estimated to occur at frequencies of less that 1 in 10^{-14} transformants.

However, as noted by Nielsen and colleagues in a review of horizontal gene transfer that acknowledges the often low frequencies of HGT, fitness must be considered, because (as with gene flow to wild relatives), genes may survive and spread if fitness is enhanced, even if initial transfer frequencies are extremely low.[33] APHIS does not consider possible fitness consequences of the transfer of rL to soil microbes. As with antibiotic resistance, lactoferrin may confer a fitness advantage to bacteria that acquire and produce it, by killing competitors.

Furthermore, recent work has demonstrated that when homologous (same sequence) DNA that is part of the transformation vector is also found in soil bacteria, HGT may occur at much higher frequencies than noted by APHIS.[34] The DNA in common between the plant-inserted vector and the bacteria can allow the transfer of adjacent DNA to the bacteria at relatively high frequencies in some cases. Importantly, although apparently not mentioned in the EA, is the presence in rL rice of multiple copies of the bacterial "backbone" of the transformation vector containing the common ColE1 "origin of replication," a kanamycin gene, and part of a lactose metabolism gene (*lacZ*), at several thousand base pairs per inserted copy.[35] The ColE1 sequences in particular

appear to be common among soil bacteria, and may thereby provide ready homology for HGT.[36] Both the nos terminator sequences and the hygromycin gene (*hpt*) also originated in bacteria. The nos terminator is found on the Ti plasmid of *Agrobacterium tumifaciens*, a common soil bacterium often found at 10^2 to 10^4 cells per gram of soil. Although it is a short DNA sequence, about 166 base pairs, it is long enough to allow HGT .[37]

The EA also ignores recent research that demonstrates HGT in humans.[38] Although not an example of HGT in the soil, it clearly demonstrates the real possibility of HGT. Artifacts were unlikely because the bacterial cells that were the source of the tested DNA were subcultured multiple times. Even though a gene commonly found in bacteria was involved in the HGT, the PCR that detected it used primers that bind to both the CP4-EPSPS gene and the plant viral promoter used to express the gene, and this chimeric gene is not found naturally in bacteria. Finally, although full copies of the target CP4-EPSPS gene were not recovered, this is likely due in part to the relatively small sample size. It is possible the CP4-EPSPS gene provided sufficient homology with the EPSPS likely found in gut bacteria to facilitate the HGT.

Additional factors determining the possibility of HGT include the natural transformation competence (natural ability for intact DNA uptake and chromosomal or plasmid integration) of soil bacteria that may contain the homologous DNA, and the possibility of expression of the gene. These factors are unknown and untested for rL to our knowledge. However, although the promoters for the rL gene are from rice, bacteria often transcribe (produce RNA) from "operons" of several genes linked to a single promoter. Therefore, it is possible, depending on the site of insertion, that a bacterial operon promoter could "read" through the plant promoter and express the gene in bacteria.

A final consideration is how the rL gene may confer a competitive advantage over soil microorganisms that may allow the propagation and spread of the gene. This is difficult to assess because although lactoferrin is antimicrobial, it may also kill the bacteria that acquire and express the gene. Furthermore, rL may need to be exported from the bacterial cell to confer an advantage, and there is no clear mechanism for this to occur. However, it may be possible that a portion of the bacterial population that normally lyse after they die could release enough lactoferrin to inhibit competing bacteria. It is also known that some bacteria are resistance to the antibiotic effects of lactoferrin, and although the known examples are human pathogens rather than soil bacteria, if similarly resistant species are found in soil and acquire the lactoferrin gene, they may develop a competitive advantage over some other microorganisms.[39]

In conclusion, the potential for HGT is likely much higher than APHIS claims, because APHIS did not consider the presence of homologous soil-bacterial DNA that may act as a "bridge" to transfer the rL gene or other plant DNA that would otherwise only be transferred at extremely low frequencies. On the other hand, the means for the rL gene to be expressed in a bacterial species and in a manner that could give that bacterial recipient a competitive advantage may only occur at very low frequencies. The overall risk from HGT is therefore difficult to determine, but APHIS has not adequately considered important factors in its EA.

Human Safety of Human-Derived Lactoferrin Produced in Rice

The EA discusses evidence to support the human safety of rL, and we consider some of these: 1) there are approximately 6 copies of the lactoferrin vector cassette in the GE rice, 2) rL is said to be denatured by cooking, 3) the physical and molecular properties of rL are "similar" to those of human lactoferrin. Under "3" are included the stability to digestion and post-translational modification of the protein. The data for all of these parameters, alone or in combination, suggest possible human health concerns.

Multiple copies of genes are often associated with gene silencing and also potentially with recombination or rearrangement. Although the genes are said to be stable, the data to support this statement are not presented. For example, in some of the earliest examples of gene silencing, pigment genes were silenced in some flowers under some environmental conditions but not others. This was easy to determine for flower color because the silencing was often observed as color sectors in individual flowers. Instability could easily be overlooked in rL rice if data from multiple seeds are combined. Therefore these data need to be carefully determined. Also, gene expression data should be gathered for a number of plants grown under different environments to determine if environment affects stability.

Another concern for multiple inserts as in Ventria's rL rice is the potential for substantial genomic rearrangements that have frequently been observed in plants transformed by biolistics. Gene insertions, especially complex insertions as with Ventria's rice, are often accompanied by thousands or tens of thousands of base pairs of scrambled genomic and vector DNA, which could affect the expression of rice genes, and which in turn could have human health or environmental consequences if ingested after contamination or transfer to weedy red rice. Several studies have demonstrated that these complex genomic DNA insertions are generally not detectable by Southern blots, the method used by Ventria to characterize the rL gene insertions.[40] Such

rearranged DNA can be responsible for unintended effects that may have either human health or environmental consequences, and have not been adequately analyzed by APHIS.

In addition, Ventria provides no data to show that expressed rL is identical in amino acid sequence to human lactoferrin. Gene sequences can be altered during the transformation process, and especially in complex transformants such as the lactoferrin rice. The transgene may be cut or sheared and re-spliced during the insertion process. If the resulting gene does not differ greatly in size from the original, for example if it differs by less than about 5 - 10% compared to the original gene, Southern blots or protein gel electrophoresis as apparently used by Ventria to examine rL will usually not detect these differences. Changes in sequence could effect properties such as digestive stability or immunogenicity. Therefore, rL protein sequences should be determined to ensure that possibly harmful sequence changes have not occurred.

Lactoferrin is said to be expressed in rice at levels comparable to those in human milk, or about 4 - 5 mg/g, and is therefore said to present no greater risk than human expressed lactoferrin. However, rL differs from human lactoferrin in that is is glycosylated differently. Plant glycosylation generally differs from human glycosylation in the absence of sialic acid residues on plant proteins, while containing xylosyl and fucosyl residues that have been associated with allergenicity.[41] Glycosylation of proteins in plants is associated with allergenicity, and differences in glycosylation between humans and other species has been associated with immunogenicity in humans. Therefore, the difference in glycosylation between the human and rL could have immunological implications and should not be assumed to be inconsequential.

Finally, the EA notes that the rL is denatured by cooking and that it is unstable in the *in vitro* digestive stability test. However, Ventria used commercial rice cookers to determine heat stability, and such cookers often have locking lids that allow some pressure to build in the cooking chamber, which cooks the rice more quickly than simple boiling. Therefore, Ventria may be using a cooking method that produces higher temperatures than boiling that may be used during home cooking. More importantly, denaturation is not synonymous with degradation. Denatured protein may in some cases be allergenic, as has been observed with the linear IgE epitopes in some cow's milk allergens or the pea allergen vicilin.[42] [43] [44] Therefore, simply demonstrating than the protein is denatured is not sufficient to ensure that it is not allergenic. The combination of altered glycosylation and lack of demonstrated degradation demonstrates that Ventria's allergenicity assessment is incomplete.

Ventria, in the Threatened and Endangered Species section citing Lonerdal, notes that human lactoferrin can be found intact in infant stool. This contradicts data summarized by Ventria that rL is very unstable in an *in vitro* gastric digestion assay that is typically used to assess potential

allergenicity. The usual explanation for the correlation observed between *in vitro* gastric digestive stability and food allergens is that stability allows the protein to reach immune tissue in the intestines, where it can cause allergy. Although CFS endorses the FAO/WHO protocols for gastric stability, which is similar to Thomas et al. used by Ventria, as an interim procedure, *in vivo* demonstration that the protein survives gastric digestion is likely more relevant. This is further evidence that the rL may be a potential allergen.

In summary, the EA does not sufficiently consider several types of data concerning rL that may have human health consequences. Therefore the EA's conclusion that rL is safe for humans is inadequately supported.

SCIENTIFIC CONCERNS WITH THE LYSOZYME EA (DOCKET 05-007-1)

In sum, the EA for lysozyme rice is inadequate for the following reasons:

- APHIS sets an inadequate 1/4 mile isolation distance to separate Ventria rice from food rice or the serious weedy relative, red rice. APHIS has underestimated gene flow from rice in the past (based on recently published research) and has seriously underestimated the ability of genetically engineered creeping bentgrass to contaminate surrounding wild relatives.[45] These and other cases are symptomatic of inadequate data on gene flow from crops. The 1/4 mile isolation distance accepted by APHIS is unlikely to ensure that gene flow will not occur.

- APHIS allows farm equipment used with Ventria's rice to be used with food rice after cleaning, despite the inability to ensure that such cleaning can remove all of Ventria's rice, which could then be transferred to food rice or contaminate the weedy rice relative, red rice. A recent report by experts on farm practices confirms that complete cleaning cannot be ensured.[46] Also, there is no requirement to clean farm machinery that was previously used on conventional rice farms prior to use on Ventria's rice, which could allow the contamination of Ventria fields by weedy red rice from a conventional farm. Because of its long seed dormancy, once in Ventria's fields, red rice could be very difficult to eradicate.

- Isolation distance must not be considered alone unless prevention of gene flow can be ensured. However, APHIS admits that minimal amounts of gene flow may occur with Ventria's rice. In conjunction with the amount of gene flow, such as by cross pollination,

the ability of the transgene to confer a competitive advantage to wild red rice must be carefully considered because enhanced competitive ability can facilitate permanent escape and spread of transgenes even when very low levels of gene flow occur. APHIS does not consider the substantial possibility that the lysozyme gene could confer a competitive advantage to wild red rice by reducing disease of red rice grain, despite the fact that lysozyme has been used in transgenic crops in previous field tests for the expressed purpose of reducing disease in those plants.

- APHIS does not consider important recent data, and thereby may seriously underestimate the possibility of horizontal gene transfer of the lysozyme gene to bacteria, and possible risk should that occur.

- Although APHIS is not qualified to determine the potential human safety risks from lysozyme rice, it has not adequately evaluated those risks.[e]

- APHIS apparently accepts evidence that lysozyme is broken down in the stomach, which would reduce its risk. However, Ventria determined that lysozyme survives for about 5 minutes in a gastric digestion assay, a similar level of stability to several known food allergens.

- APHIS apparently accepts that lysozyme is destroyed by cooking despite inadequate description of testing methods. It may be that the protein is only denatured, which does not assure that a protein will not be an allergen.

Environmental Safety of Human-Derived Lysozyme Produced in Rice

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[e] APHIS has consulted with a representative from FDA to help in its human safety determination, however, FDA has not completed its own human safety assessment, so this assistance is unlikely to be adequate.

Gene Flow from Lysozyme Rice to the Wild Relative, Red Rice

A critical issue for the environmental safety of pharmaceutical crops is gene flow to wild crop relatives. As the EA notes, there appears to be one wild sexually compatible relative of cultivated rice in the U.S., annual red rice, *Oryza sativa* ssp *sativa*. Cultivated rice can sometimes become weedy as well, and can grow as a volunteer feral plant in rice growing areas. Red rice is a serious weed of rice, and is reported to be found in Missouri.[47] In fact, some sources consider it to be the most serious rice weed in Missouri.[48] Red rice often readily hybridizes with, and produces fertile progeny with, cultivated rice that are often more vigorous that either parent, which could enhance survival after gene flow at low frequencies.[49] Furthermore, APHIS likely underestimates the potential for gene flow because it considers rice to be primarily self-pollinating. Although true, this assessment ignores data by Langevin and colleagues who found hybridization of red rice with a cultivar of commercial rice at 52%, which indicates the potential of a high rate of outcrossing under at least some circumstances.[50]

If the rLY gene contaminates red rice, it could spread well beyond Ventria's field test sites. Furthermore, because red rice seed has extended dormancy, and can survive in the soil for at least 6 or 7 years, once red rice is contaminated, it would be very difficult to eradicate or prevent further spread.

The EA discloses several permit conditions intended to reduce gene flow to either red rice or cultivated rice. The primary means of reducing gene flow is to require a separation distance of at least 1/4 mile between the Ventria field test and either cultivated rice or red rice. Ventria further claims that there have been no rice fields within 10 miles of the field test site. This estimated separation is based on a personal communication of a University of Missouri extension specialist, with no indication of how this estimation was derived. As such, it should not be given much weight. In addition, as noted in the EA, it cannot be ensured that rice will not be grown closer to the field test site, so the 1/4 mile requirement must be the standard used for assessing possible gene flow. Furthermore, as noted in the EA, Ventria intends to expand its acreage over time, and to grow in Mississippi and Cape Girardeau counties as well as the current Scott County (EA, page 44, TES Worksheet). Northwest Missouri State University is collaborating with Ventria and its president, Dean Hubbard, has stated that eventually Ventria expects to increase cultivation of its pharmaceutical crops to 25,000 acres.[51] The latter acreage is 39 square miles, which clearly could make ensuring separation distances of greater than 1/4 mile improbable in these counties.

In addition, Ventria claims that the counties discussed in the field test application grow a total of 500 acres of rice (EA page 44, TES worksheet), all in Scott county. To the contrary, recent preliminary state agricultural data for 2003 for Scott county indicate 1200 acres of rice (data for Scott County alone was not previously recorded, but has been since 2003 due to increasing rice acreage). For the three other counties of Cape Girardeau, Mississippi, and Bolinger, 1400 acres were grown in 2003 (individual county data not available). Therefore, the three counties where Ventria plans on growing rLY rice, plus Bolinger, grew about 2,600 acres of rice in 2003, compared to about 300 acres in 1996, or an increase of almost 9 fold in 8 years.[52] [53] Taken together, these data indicate a trend of increasing food rice cultivation in these counties, which may encroach on Ventria's rice significantly if it continues, and already questions Ventria's isolation assumptions. In addition, surrounding counties grow large amounts of rice, for example, Stoddard County grows about 58,000 acres of rice. For all of these reasons, the 1/4 mile separation distance to cultivated and red rice must be used as the *de facto* separation distance.

On page 17 of the EA, APHIS claims that because rice pollen survives for only a few minutes (5 to 20 minutes is often cited), the 1/4 mile separation from food rice or red rice would reduce gene flow to "*de minimus*" levels. The EA does not define what is meant, in practice, by "*de minimus.*" Unfortunately, APHIS assurances about adequate confinement distance have been repeatedly proven wrong, primarily because data on low levels of gene flow that could be important when considering pharmaceutical rice and pollination of wild relatives have generally not been available. For example, APHIS previously required only a 10 foot separation distance for GE rice, based on seed purity standards for conventional breeding. APHIS then increased this distance to 100 or 200 feet for pharmaceutical rice. However, recent research indicates that gene flow may occur at 110 meters, between a quarter and a third of the 1/4 mile distance required separation distance.[54]

Furthermore, the maximum distance for rice pollen dispersal estimated in this research was based on only a few measurements in calm weather, and was extrapolated from those measurements using regression analysis, and where the largest pollen source was only 72 m^2, compared to the reported 204 acres for the Ventria field tests. This method of distance estimation is unlikely to adequately account for either the leptokurtic distribution often seen in pollen flow measurements (i.e., instead of pollen dispersal diminishing in a regular manner to zero over distance, low levels of pollen can be found at very long distances from the source), nor account for weather conditions that could substantially increase pollen dispersal, for example thunder storms, high winds, or air turbulence. The authors note that their suggestion of a 110 meter isolation distance is based on "normal weather conditions." They also note that: "Undoubtedly, a detailed study is

needed to find out what the influences of other weather parameters are on the distribution of rice pollen flow under normal field conditions."[55]

The approach of limited incremental increases in isolation distance compared to previous standards, as proposed by Ventria, does not address the fundamental lack of sufficient data on pollen dispersal. Because few experiments have been carried out that include a range of environmental conditions, current data cannot be considered to represent maximum distances for gene flow. The most recent example of APHIS underestimation of pollen dispersal was an experiment with GE creeping bentgrass where gene flow was found at the greatest distances measured - about 13 miles from a field test - which was miles farther than expected based on the much more limited confinement requirements that APHIS placed on that field test.[56] Similarly, although APHIS typically requires a 1/4 mile isolation distance for genetically engineered (non-pharmaceutical) canola, recent experiments show small amounts of gene flow at 3 kilometers.[57]

All of these distances should be considered preliminary, because various conditions may affect the results by affecting the rate of pollen travel and survival time. Factors such as wind speed, air turbulence, ambient temperature, cloud cover (UV light penetration), and humidity may all affect the distance over which gene flow may occur. In addition, the relative sizes of the pollen source (Ventria rice fields) and red rice stands is likewise important, because a large pollen source and relatively small number of accepting plants may increase gene flow.[58] [59] Although APHIS inappropriately does not reveal the size of the field test, press reports citing Scott Deeter of Ventria indicate that their field test applications (presumably for lactoferrin and lysozyme) are for about 200 acres.[60] It is therefore likely that the source of rLY pollen will likely be much greater than the size of red rice stands.

APHIS also does not consider observations of possible pollination of rice by insects. For example a small increase in outcrossing of rice has been observed when honey bees are present.[61] Clearly rice is primarily wind pollinated, but low levels of insect pollination need to be considered. Honey bees often forage over distances greater than 1/4 mile, sometime over several miles. Therefore, bee or other insect pollination could be important for gene flow to red rice.

Data on gene flow distances typically follow a skewed leptokurtic distribution. Graphs of gene flow distances usually show a very long distribution "tail," whereby low levels of gene flow occur over very long distances without falling to zero (at least, not within the distances observed

in the experiments).[62] [f] The skewed distribution means that APHIS cannot simply examine most current, limited, data and extrapolate safe isolation distances.

Another possible means of gene flow could occur from farm machinery. In particular, machines with complex internal compartments not readily accessible from the outside may harbor small amounts of seed, even when cleaned. APHIS requires machinery used on biopharm crops to be "dedicated' to that crop. However, APHIS definition of "dedicated" allows the farm equipment to be used on conventional food crops after cleaning. In the case of Ventria, machinery such as planters, and especially harvesters, may be used with food rice after they are used for rLY rice after cleaning. Ventria remarks in the EA that it will clean its equipment according to APHIS standards prior to use with food rice. A recent review of biopharm crops by experts knowledgeable about farm equipment concluded that such equipment cannot usually be cleaned carefully enough to ensure that biopharm seed will not be co-mingled with food seed.[63] If that occurs, such seed could either end up in the food supply, or in seed used for planting future rice crops. In the latter case, rLY rice would be planted unsuspectingly, and could thereby be perpetuated in the food supply, or contaminate red rice.

There is also no requirement to clean farm equipment *prior* to use on Ventria's fields. This allows the possibility for red rice to be introduced to Ventria's fields from other rice fields. Red rice growing in Ventria's fields would be cross pollinated by Ventria's rice, transferring the rLY gene. And because of its very long seed dormancy period, such rLY red rice could easily evade volunteer rice control protocols, and emerge years after a particular field is no longer used by Ventria. And rLY red rice, once in Ventria's fields, would not only be extremely difficult or impractical to eradicate, it may eventually escape in machinery or by other means.

Also morphological convergence between red rice and cultivated rice has been reported in some cases. This makes the detection of red rice growing among cultivated rice more difficult.[64] [65] This could make assessment of the proximity of red rice more difficult or inaccurate, and increase the possibility that machinery, such as combines, could unwittingly harvest red rice prior to use in Ventria's fields.

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[f] Recent experiments by Rieger et al. show a more random distribution, but this may have occurred due to the combination of data from several sites. In any case, those data also demonstrate higher than expected gene flow at longer distances than would otherwise be expected.

Finally, APHIS does not adequately consider the movement of seed by birds, mammals, or water. As discussed in the following section, low frequency events may be sufficient to cause permanent escape of the rLY gene to red rice if the fitness of the wild species is enhanced. Therefore, the evaluation by APHIS on dispersal by birds, mammals or flooding is inadequate because it does not consider potential impact of low levels of initial gene flow. In particular, due to the relatively short required isolation distance of 1/4 mile, incidental transfer, such as rice grains temporarily lodged in the feathers of waterfowl or the fur of mammals, could occur. Similarly, the screens and grates described by APHIS to prevent transfer by water can become clogged or overflow. Although the EA (page 16) claims that the nearest body of water is 4 miles from the field test site, elsewhere, page 44 of the EA notes that "The nearest non-agricultural water is more than one mile from the field." Such a location may allow the growth of volunteer rice for long enough to transfer to red rice.

APHIS's unsupported claim that the lack of observer volunteer rice at the edges of rice fields is "strong evidence" that transfer of rice does not occur is not credible without any discussion about how frequently anyone looks for such volunteer rice.

In summary, several routes of possible gene flow to red rice or cultivated food rice exist that have not been adequately considered by APHIS. The assertion that gene flow would occur only at *de minimus* levels cannot be considered credible without better data, and absent consideration of the possible fitness consequences of gene flow to red rice, considered in the following section.

Failure to Evaluate the Potential Contribution of the Lysozyme Gene to the Fitness and Weediness of Red Rice

Isolation distance must not be considered separately from the ability of the transgene(s) to survive after gene flow occurs. Low levels of gene flow can lead to the permanent escape of the transgene if it confers a selective advantage to a wild relative, in this case, red rice.[g] Put differently, a gene that confers a fitness advantage may persist and spread in a wild relative where a gene that is disadvantageous, may not survive or spread.[66] Therefore, fitness, or the competitive advantage conferred to a wild relative by a transgene, is of fundamental importance in determining the possibility of transgene escape unless the absence of gene flow can be ensured. As has been noted, APHIS does not ensure that gene flow will not occur, but only that

[g] A fitness-neutral transgene may also survive in red rice, but at lower numbers and with less likelihood.

it may occur at *de minimus* levels. However, without properly considering the possible fitness conferred to red rice by the lysozyme gene, APHIS cannot properly determine what a *de minimus* level of gene flow may be.

Unfortunately, APHIS does not adequately consider the ability of the lysozyme gene to persist and spread if it escapes to its wild relative, red rice. In fact, APHIS apparently does not even consider the possibility that the lysozyme gene may confer a selective advantage to red rice. APHIS merely states that lysozyme (or hygromycin) "is [not] expected to alter the susceptibility of the transgenic rice plants to disease or insect damage." (EA, page 15) This a fundamental shortcoming of the APHIS argument for the safety of lysozyme rice, and in combination with inadequate analysis of gene flow and the 1/4 mile isolation distance, constitutes a major failing of the APHIS risk assessment.

In particular, APHIS does not assess the possibility that the well known antimicrobial properties of lysozyme may confer a competitive advantage to red rice. Resistance to diseases or insects are properties that the National Academy of Sciences (NAS) and others consider to potentially confer a competitive advantage to wild crop relatives, by increasing survival compared to plants that do not possess the gene.[67] The NAS stated that: "Generally, if an allele confers a fitness advantage - once introduced into a population - it is expected to increase in frequency, even if it is introduced only once."

One of the primary commercial applications of lysozyme is as an anti-microbial. APHIS acknowledges in the EA that lysozyme has been noted to have antimicrobial properties. It is especially remarkable that APHIS dismisses the possibility that lysozyme could confer disease resistance to rice or red rice, because nine field tests have been approved by APHIS for four crops containing lysozyme specifically intended to mitigate four bacterial plant diseases.[68] In addition, the gene is expressed at a very high level compared to most transgenes, approximately 5 mg/g. This is about 20 fold higher than found in human milk (about 0.250 mg/ml, according to Ventria, where 1 ml ~ 1 g) where it confers antimicrobial properties. For example, by comparison, Bt Cry proteins are typically expressed at microgram/g levels, typically several hundred to several thousand fold less than for lysozyme in rice.

APHIS repeats in its EA the same absence of rigor in determining the possible contribution of a disease resistance gene to fitness that was strongly criticized by the NAS in 2002, when APHIS failed to adequately analyze the possible consequences of virus-resistance gene flow to wild squash.[69] In that case, APHIS conducted an analysis of possible impacts of gene flow to wild squash, but the NAS found the analysis inadequate. In the case of lysozyme, not even a cursory

analysis has been conducted. APHIS merely mentions that no differences in disease susceptibility of transgenic rLY rice were observed in previous field tests. However, such an analysis has little value if relevant pathogens were not present, or without discussion about how rigorous the observations were. Those previous field tests also were not conducted in Missouri, which has a somewhat different spectrum of rice diseases than states in other parts of the country (California or Hawai'i), where previous rLY field trials have taken place. It is also possible that pesticides were applied to reduce bacterial diseases, which would have made disease observations of little value.

As discussed in the NAS report, a proper analysis should consist of determining the susceptibility of red rice to rice seed diseases, prevalence of such diseases in Missouri, susceptibility of rLY rice after inoculation with the pathogen, etc. There is no evidence that any such studies were conducted.

Because APHIS provides no explanation for its apparent lack of concern about possible competitive advantages conferred to red rice by lysozyme, we can only speculate as to the lack of adequate analysis. One reason may be that lysozyme is said to be expressed only in the seed of rice, rather than in the entire plant, as is the case with many other transgenes. Such reasoning would be misplaced. For example, the American Phytopathological Society lists several diseases of rice kernels, which may affect fecundity.[70] And fecundity, including survival of seed, is a major fitness component. For example, recent research found that a Bt Cry protein substantially increased survival of wild sunflower seed by protection against several lepidopteran (moth) pests, allowing more progeny to be produced.[71]

Seed often contains proteins that confer resistance to insects or diseases. These proteins include trypsin inhibitors, lectins, and alpha-amylases that inhibit insects, and pathogenesis related (PR) proteins such as chitinases and beta-glucanases that inhibit diseases.72 73 74 If these proteins did not confer a significant advantage to the plant, they would likely not be produced because of the metabolic costs involved. However, although these endogenous pest protections are effective against many insects and diseases, the ones produced in rice seeds are not effective, by definition, against the pathogens and insects that harm rice. Therefore, it is clear that an antimicrobial protein like lysozyme, that may confer resistance to diseases that are not controlled by endogenous antimicrobial substances in the rice grain, may confer a substantial competitive, selective, advantage to red rice.

Finally, the likelihood that gene flow will occur from a single field test is lower than for continuing field tests or commercial production. Ventria has expressed the intention of

continuing these field tests indefinitely and expanding the acreage dramatically. Under such circumstances, contamination of food rice and red rice becomes more likely.

Horizontal Gene Transfer to Soil Bacteria from Lysozyme Rice

APHIS does not consider horizontal gene transfer (HGT) to soil microorganisms to be a significant risk. APHIS does not evaluate important research or fitness concerns when evaluating the possibility of HGT. APHIS cites experimental data indicating that HGT occurs at exceedingly low frequencies, often undetectable, and sometimes estimated to occur at frequencies of less that 1 in 10^{-14} transformants.

However, as noted by Nielsen and colleagues in a review of horizontal gene transfer that acknowledges the often low frequencies of HGT, fitness must be considered, because (as with gene flow to wild relatives), genes may survive and spread if fitness is enhanced, even if initial transfer frequencies are extremely low.[75] APHIS does not consider possible fitness consequences of the transfer of rLY to soil microbes. As with antibiotic resistance, lysozyme may confer a fitness advantage to bacteria that acquire and produce it, by killing competitors.

Furthermore, recent work has demonstrated that when homologous (same sequence) DNA that is part of the transformation vector is also found in soil bacteria, HGT may occur at much higher frequencies than noted by APHIS.[76] The DNA in common between the plant-inserted vector and the bacteria can allow the transfer of adjacent DNA to the bacteria at relatively high frequencies in some cases. rLY rice may have approximately two copies of the bacterial "backbone" of the transformation vector, which may contain the common ColE1 "origin of replication," and other sequences of bacterial origin that may be several thousand base pairs per inserted copy.[77] The ColE1 sequences in particular appear to be common among soil bacteria, and may thereby provide ready homology for HGT.[78] Both the nos terminator sequences and the hygromycin gene (*hpt*) also originated in bacteria. The nos terminator is found on the Ti plasmid of *Agrobacterium tumifaciens*, a common soil bacterium often found at 10^2 to 10^4 cells per gram of soil. Although it is a short DNA sequence, about 166 base pairs, it is long enough to allow HGT [79]

The EA also ignores recent research that demonstrates HGT in humans.[80] Although not an example of HGT in the soil, it clearly demonstrates the real possibility of HGT. Artifacts were unlikely because the bacterial cells that were the source of the tested DNA were subcultured multiple times. Even though a gene commonly found in bacteria was involved in the HGT, the

PCR that detected it used primers that bind to both the CP4-EPSPS gene and the plant viral promoter used to express the gene, and this chimeric gene is not found naturally in bacteria. Finally, although full copies of the target CP4-EPSPS gene were not recovered, this is likely due in part to the relatively small sample size. It is possible the CP4-EPSPS gene provided sufficient homology with the EPSPS likely found in gut bacteria to facilitate the HGT.

Additional factors determining the possibility of HGT include the natural transformation competence (natural ability for intact DNA uptake and chromosomal or plasmid integration) of soil bacteria that may contain the homologous DNA, and the possibility of expression of the gene. These factors are unknown and untested for the rLY gene to our knowledge. However, although the promoters for the rLY gene are from rice, bacteria often transcribe (produce RNA) from "operons" of several genes linked to a single promoter. Therefore, it is possible, depending on the site of insertion, that a bacterial operon promoter could "read" through the plant promoter and express the gene in bacteria.

A final consideration is how the rLY gene may confer a competitive advantage over soil microorganisms that may allow the propagation and spread of the gene. This is difficult to assess because although lysozyme is antimicrobial, it may also kill the bacteria that acquire and express the gene. Furthermore, it is likely that rLY would have to be exported from the bacterial cell to function, and there is no clear mechanism for this to occur. However, it may be possible that a portion of the bacterial population that normally lyse after they die could release enough lysozyme to inhibit competing bacteria. It is also possible that some soil bacteria are resistance to the antibiotic effects of lysozyme, and if they acquire the lysozyme gene, they may develop a competitive advantage over some other microorganisms.

In conclusion, the potential for HGT is likely much higher than APHIS claims, because APHIS did not consider the presence of homologous soil-bacterial DNA that may act as a "bridge" to transfer the rLY gene or other plant DNA that would otherwise only be transferred at extremely low frequencies. On the other hand, the means for the rLY gene to be expressed in a bacterial species and in a manner that could give that bacterial recipient a competitive advantage may only occur at very low frequencies. The overall risk from HGT is therefore difficult to determine, but APHIS has not adequately considered important factors in its EA.

Human Safety of Human-Derived Lysozyme Produced in Rice

The EA discusses evidence to support the human safety of rLY, and we consider some of these: 1) there are approximately two copies of the lysozyme vector cassette in the GE rice, 2) rLY is said to be destroyed by cooking, 3) the physical and molecular properties of rLY are "similar" to those of human lysozyme. Under "3" are included the stability to digestion and post-translational modification of the protein. The data for all of these parameters, alone or in combination, suggest possible human health concerns.

Multiple copies of genes are often associated with gene silencing and also potentially with recombination or rearrangement. Although the genes are said to be stable, the data to support this statement are not presented. For example, in some of the earliest examples of gene silencing, pigment genes were silenced in some flowers under some environmental conditions but not others. This was easy to determine for flower color because the silencing was often observed as color sectors in individual flowers. Instability could easily be overlooked in rLY rice if data from multiple seeds are combined. Therefore these data need to be carefully determined. Also, gene expression data should be gathered for a number of plants grown under different environments to determine if environment affects stability.

Another concern for multiple inserts as in Ventria's rLY rice is the potential for substantial genomic rearrangements that have frequently been observed in plants transformed by biolistics. Gene insertions, especially complex insertions as with Ventria's rice, are often accompanied by thousands or tens of thousands of base pairs of scrambled genomic and vector DNA, which could affect the expression of rice genes, and which in turn could have human health or environmental consequences if ingested after contamination or transfer to weedy red rice. Several studies have demonstrated that these complex genomic DNA insertions are generally not detectable by Southern blots, the method used by Ventria to characterize the rLY gene insertions.[81] Such rearranged DNA can be responsible for unintended effects that may have either human health or environmental consequences, and have not been adequately analyzed by APHIS.

In addition, Ventria provides no data to show that expressed rLY is identical in amino acid sequence to human lysozyme. Gene sequences can be altered during the transformation process, and especially in complex transformants such as the lysozyme rice. The transgene may be cut or sheared and re-spliced during the insertion process. If the resulting gene does not differ greatly in size from the original, for example if it differs by less than about 5 - 10% compared to the original gene, Southern blots or protein gel electrophoresis as apparently used by Ventria to

examine rLY will usually not detect these differences. Changes in sequence could effect properties such as digestive stability or immunogenicity. Therefore, rLY protein sequences should be determined to ensure that possibly harmful sequence changes have not occurred.

The EA notes that the rLY is destroyed by cooking and that it is unstable in the *in vitro* digestive stability test. However, Ventria used commercial rice cookers to determine heat stability, and such cookers often have locking lids that allow some pressure to build in the cooking chamber, which cooks the rice more quickly than simple boiling. Therefore, Ventria may be using a cooking method that produces higher temperatures than boiling that may be used during home cooking. Ventria also claims that rLY cannot be detected by Western (immuno-) blot after cooking. However, Ventria does not disclose the source of antibodies used to detect rLY in the Western blots. If the antibodies were raised to "native" lysozyme, they may primarily bind to conformational epitopes and may not detect linear epitopes of denatured lysozyme. Therefore, without knowing whether the antibodies used can bind denatured rLY, it cannot be assumed that lack of detection on a Western blot reveals degradation as opposed to denaturation. Denaturation is not synonymous with degradation. Denatured protein may in some cases be allergenic, as has been observed with the linear IgE epitopes in some cow's milk allergens or the pea allergen vicilin.[82 83 84] Therefore, simply demonstrating than the protein is denatured is not sufficient to ensure that it is not allergenic. APHIS should require experiments that clearly determine whether rLY is degraded or denatured, and if only denatured, what its allergenic potential may be.

Ventria asserts that rLY is undetectable after five minutes in an *in vitro* gastric digestion assay that is typically used to assess potential allergenicity. Many food allergens are somewhat or very stable in this assay while most non-allergenic food proteins are very unstable. The usual explanation for the correlation observed between *in vitro* gastric digestive stability and food allergens is that stability allows the protein to reach immune tissue in the intestines, where it can cause allergy. Although Ventria cites this data in support of non-allergenicity, several important food allergens have been stable for only between about two and 15 minutes in this assay.[85] Therefore, stability of five minutes, an intermediate value, may indicate potential allergenicity.

In summary, the EA does not sufficiently consider several types of data concerning rLY that may have human health consequences. Therefore the EA's conclusion that rLY is safe for humans is inadequately supported.

CONCLUSIONS FOR BOTH EA'S

In sum, the lactoferrin and lysozyme rice EAs are inadequate under NEPA and should be revised to address the issues we raise herein. Then APHIS should put them out again for further public comment before any decision is made on the Ventria permit applications. Alternatively, full environmental impact statements should be prepared.

We look forward to your written responses to each of these comments individually and to further participating in the NEPA compliance process. For further information on these comments, please contact either of us listed below.

Sincerely,

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Enclosures (incorporated by reference)

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77. Ventria does not provide an adequate description of all DNA inserted into the rLY plants, but the data submission for lactoferrin rice to FDA by Ventria discloses the bacterial vector backbone and other sequences. It is possible that the same vector was used to make rLY plants, because companies will often use such successful vectors to make many different GE crops, and because the particular bacterial backbone, from the pUC19 plasmid, is perhaps the most commonly used of all for such purposes. For lactoferrin, see: Ventria Bioscience, "Safety, Compositional and Nutritional Aspects of LF164 Rice Transformational Event, voluntary data submission to FDA, Center for Food Safety and Applied Nutrition, designated BNF 082, Nov. 24, 2003

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Comments on APHIS Environmental Assessment for Permit Application No. 04-302-01r for Outdoor Cultivation of Rice Expressing a Novel, Recombinant Human Lactoferrin

Submitted to USDA's Animal and Plant Health Inspection Service
Docket No. 05-006-1

by

Friends of the Earth

March 25, 2005

Introduction

Friends of the Earth (FoE) appreciates the opportunity to comment on the environmental assessment conducted by APHIS on Permit Application No. 04-302-01r, submitted by Ventria Bioscience, for cultivation of rice genetically engineered to produce a novel recombinant version of human lactoferrin on 100 acres in Scott County, Missouri. FoE urges the USDA to ***deny*** this permit application because granting it would pose unacceptable risks to consumers, the environment, the economic welfare of farmers and the rice and food industries.

FoE supports a moratorium on the cultivation of food crops genetically engineered to produce pharmaceutical and industrial compounds; at most, only non-food crops grown in carefully contained conditions should be permitted for production of recombinant pharmaceuticals or industrial compounds. Any measures we may recommend in the following comments to reduce gene flow, monitor for environmental impacts, etc. are not to be understood as an endorsement of biopharming if those measures are implemented, but rather only as second-best measures to mitigate certain impacts of biopharming.

Our comments consist of two reports published by FoE on Ventria and its pharmaceutical rice varieties, plus a scientific article on therapeutic uses and hazards of recombinant human lactoferrin by Dr. Eugene Weinberg. The reports are based on extensive research into all aspects of this venture into biopharming: contamination risk, potential human health and environmental impacts, regulatory inadequacies, economic impacts on rice farmers and the food industry from contamination episodes, and finally the failure of biopharming to live up to its promise. One report – *Pharmaceutical Rice in Missouri* – is more directly relevant to issues raised in the environmental assessment, particularly the potential for contamination and the hyping of this unproven technology. The second, *Pharmaceutical Rice in California*, should be consulted for its greater detail on the issue of pharmaceutical gene flow in general, the potential human health impacts of Ventria's recombinant human proteins, the adverse environmental impacts from transfer of Ventria's pharmaceutical traits to red rice, and the blatant regulatory inadequacies with respect to pharm crops in general and Ventria's rice in particular. The reports and scientific article are preceded by a March 17th letter to Undersecretary William Hawks requesting that APHIS deny Ventria's permit applications, and a brief critique of the evidentiary basis of APHIS regulatory action.

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March 17, 2005

Mr. William Hawks, Undersecretary
U.S. Department of Agriculture
1400 Independence Avenue, SW
Washington DC 20250

RE: Pharmaceutical Rice in Missouri

Dear Mr. Hawks,

We are writing to request that the U.S. Department of Agriculture (USDA) reject three applications by Ventria Bioscience to grow genetically engineered, pharmaceutical-producing rice varieties in southeast Missouri this year (permit application numbers 04-302-01r, 04-309-01r and 05-004-01r).

Our reasons for this request are detailed in the enclosed briefing paper, *Pharmaceutical Rice in Missouri*. In brief:

1) Despite improvements in USDA regulation of pharmaceutical food crops over the past few years, such measures can only ***reduce***, not ***prevent***, contamination of the food supply with experimental plant-made pharmaceuticals. The proposed site of these trials – Scott County, Missouri – is on the northeastern edge of the country's most productive rice-growing region, increasing the potential for contamination.

2) Ventria's products are potentially hazardous to consumers and the environment because they have pharmaceutical and pesticidal properties; yet neither the proposed field trials nor the pharmaceutical proteins have been reviewed by the Food and Drug Administration or the Environmental Protection Agency. The environmental assessments of the proposed field trials conducted by USDA's Animal and Plant Health Inspection Service (APHIS) are no substitute for a full environmental impact statement by USDA as well as formal reviews by FDA and EPA.

3) There is a *de facto* zero tolerance standard in place for plant-made pharmaceuticals in the food supply. Therefore, even low-level "adventitious presence" of Ventria's rice-grown pharmaceuticals in the food chain poses unacceptable regulatory and market-based liability to farmers and food companies.

We urge you to reject these applications in order to protect Missouri farmers, consumers, and the rice & food industries from harm. These permits could be granted as early as the end of March. Therefore, we would appreciate your prompt attention to this matter. Please let us know what action you decide to take.

Sincerely yours,

Bill Freese
Research Analyst
Friends of the Earth

Joseph Mendelson
Legal Director
Center for Food Safety

Kerry-Ann T. Powell
Staff Attorney
U.S. Public Interest Research Group

cc: Fred Ferrell, Director, Missouri Department of Agriculture
Jay Nixon, Missouri State Attorney General
Matt Blunt, Governor, State of Missouri
Stuart Proctor, President & CEO, USA Rice Federation
Mark Nelson, Vice President, Scientific and Regulatory Affairs, Grocery Manufacturers of America
Jeffrey Barach, Vice President, Special Projects, Scientific and Regulatory Affairs, National Food Processors Association

Evidentiary Basis

Friends of the Earth believes that regulatory assessments of genetically engineered (GE) crops should be conducted with the goal of ensuring protection of the public and the environment from harm, not increasing public confidence in plant biotechnology. If the former is done well, the latter will follow as a matter of course. The converse is not true. Therefore, attempts to make "increasing public confidence" in the regulated article an independent goal of regulation are deeply misguided and should be dropped.

Protecting the public and the environment from harm requires a thorough, balanced assessment that should be conducted in a scientifically rigorous manner. Due attention should be given to various aspects of the assessment according to their importance. Assertions should be documented, whenever possible with published, peer-reviewed literature. Casual observations must not be allowed to substitute for well-founded arguments and documented assertions. The methodology of tests or experiments whose results are cited as a basis for claims of safety must be examined critically; the methodology should be detailed or referenced so that others (i.e. public reviewers) may review it. Any undocumented assertions or observations should be either entirely dismissed until supporting evidence is provided, or at least viewed with extreme skepticism as most likely untrue. Uncertainty at any level should invoke application of the precautionary principle.

Both Ventria and APHIS often ignore these basic principles of scientific procedure in their respective safety and environmental assessments. While this EA represents an improvement over the handful of brief, mechanical, boilerplate EAs conducted by APHIS on GE pharmaceutical crop trials from 1991 to 1998,[1] it is still far from meeting the standards of scientific scholarship. This environmental assessment would be rejected by any reputable scientific journal for these inadequacies. It would be returned to the authors with numerous questions for clarification and requests for documentation. Therefore, this EA does not provide an adequate basis for a finding of no significant impact for Ventria's prospective field trial of rice expressing recombinant human lactoferrin (rhLf).

Pharmaceutical Rice in Missouri

Ventria Bioscience: From California to Missouri

Since 1997, Ventria Bioscience or its predecessor Applied Phytologics has been conducting outdoor field trials of rice genetically engineered to produce experimental pharmaceuticals in California's rice-growing Central Valley.[1] These pharmaceutical compounds include artificial versions of the human milk proteins lactoferrin and lysozyme as well as the blood protein, serum albumin. Proposed uses for the whole rice and/or extracted pharmaceuticals include poultry

[1] For an analysis, see: Freese (2002). "Manufacturing Drugs and Chemicals in Crops: Biopharming Poses New Threats to Consumers, Farmers, Food Companies and the Environment," Friends of the Earth, July 2002, section 6. www.foe.org/biopharm/.

feed, treatment of diarrhea, infant food and topical wound treatment. Permitted field trial acreage grew from several acres in 1997 to 93 acres in 2003. Ventria's bid to begin production on 120 acres in California in 2004 was blocked by California and federal authorities due mainly to the concerns of rice growers that these pharmaceuticals would contaminate food-grade rice and thereby lead to export market rejection.[2]

Having failed in California, Ventria decided to relocate. The company reportedly approached eight states, but Missouri offered the best deal – a subsidy package of $30 million to fund construction of facilities at Northwest Missouri State University at Maryville and $5 million more "to finance Ventria's operating costs before profits are made."[3] Ventria has applied to USDA for three permits to grow up to 204.5 acres of pharmaceutical rice in rice-growing southeastern Missouri in 2005, and has plans to grow up to 28,000 acres in the future[4] (see Attachment 1 for map).

Experimental and Unproven

Pharmaceutical crops such as Ventria's rice represent an ***experimental*** and ***unproven*** application of biotechnology. Not a single "plant-made pharmaceutical" (PMP) has been approved by the U.S. Food and Drug Administration (FDA), despite huge infusions of capital and government subsidies, numerous clinical trials, and field trials dating back to 1991. Ventria itself does not have a single commercial pharmaceutical product.[5]

> *"In addition, we recognize the possibility of the inadvertent introduction of LF164 [pharmaceutical rice] at low, adventitious levels into commercial rice varieties."*[27]
>
> Delia R. Bethell, Vice President, Ventria Bioscience, in letter to FDA's Robert Martin, 11/24/03

Contamination is Likely

The federal government has a "zero tolerance" standard for PMPs in food. Yet scientists and agronomists agree that it is virtually impossible to keep PMPs from entering the food and feed supply when food crops are engineered to produce these compounds. The National Academy of Sciences warned of this risk in two recent reports.[6] The editors of a leading journal in the field, *Nature Biotechnology*, recently compared growing drugs in food crops to a pharmaceutical manufacturer "packaging its pills in candy wrappers or flour bags or storing its compounds or production batches untended outside the perimeter fence."[7] These concerns are validated by numerous episodes in which conventional crops and ***certified seed stocks*** have become contaminated with transgenic traits.[8] A recent report from the Union of Concerned Scientists, with contributions from leading scientists, also suggests that PMPs in food crops cannot be kept out of the food supply.[9] That these are not idle warnings is illustrated by events that occurred just over two years ago.

> *"...it is possible that crops transformed to produce pharmaceutical or other industrial compounds might mate with plantations grown for human consumption, with the unanticipated result of novel chemicals in the human food supply."*[16]
>
> National Academy of Sciences, 2002

In two incidents in 2002, pharmaceutical corn adulterated 500,000 bushels of soybeans in Nebraska and 155 acres of corn in Iowa; the adulterated soy was seized and destroyed, the corn burned, costing millions of dollars.[10] Continued cultivation of Ventria's rice could have a similar outcome... especially in Missouri.

Risk of Contamination in Missouri and Arkansas

Contamination is a significant threat in Missouri and Arkansas, for at least seven reasons:

1) **Food-grade rice in vicinity:** Ventria plans to grow its rice in three counties (Cape Girardeau, Scott and Mississippi) on the northeastern edge of one of the nation's most productive rice-growing areas (Missouri Bootheel / northeastern Arkansas).[11] Attachment 2 shows the acreage of rice harvested in 15 counties of this region. Note that the acreage in each of five counties exceeds 50,000 acres. This includes one county – Stoddard – situated directly adjacent to Scott County, where one of the field trials would take place. Scott County itself had over 1,000 acres of rice in 2004.[12]

"... the food industry must have a '100% protection standard' against any contamination of the food supply. If this standard cannot be met, we will vigorously oppose the use of food or feed crops as 'factories' for pharmaceutical and industrial chemical products. We can demand nothing less for our consumers."[28]

Dr. Rhona Applebaum, National Food Processors Association, 2/6/03

2) **Pharmaceuticals in red rice:** Ventria's rice could spread its pharmaceuticals to weedy red rice, which is the most troublesome weed in Missouri and Arkansas,[13] through cross-pollination. Rice and red rice hybridize easily, and the more vigorous weedy hybrids are already a significant and costly problem for Missouri and Arkansas rice growers. Once they "introgress" into red rice, Ventria's pharmaceuticals, which have antifungal and antimicrobial properties, might lend weedy hybrids a fitness boost, making them tougher to eradicate.[14] The seriousness of this issue is indicated by a major research initiative underway at the University of Arkansas examining the potential for genetically-engineered traits (particularly herbicide-resistance) to enter red rice and exacerbate weed infestation problems.[15]

3) **Seed dispersal via animals:** Birds and other animals are major rice pests in southeastern Missouri, and could disperse pharmaceutical rice through ingestion and defecation of viable grains. In fact, a USDA study recently reported a population of over 3 million red-wing blackbirds (voracious rice eaters) roosting in rice fields near Sikeston, MO[16] (see Attachment 2). Migrating geese and ducks also frequent Missouri rice fields and consume large quantities of rice. Geese have inefficient digestive systems and frequently defecate poorly digested foodstuff, and so could spread viable pharm rice grains large distances.[17] Red rice has been observed to sprout from grains in the craws of geese after the birds are downed by storms or hunters, and the same thing might occur with pharm rice.[18] Neither federal regulators nor Ventria took adequate measures to stop dispersal of pharm rice via bird or animal in California, and are unlikely to do so in Missouri.[19]

"The draft proposal is light on some details, including how Ventria will prevent birds from spreading its rice; what constitutes "proper" disposal of rice plants; and whether the company will notify nearby growers."[19]

Sacramento Bee, 1/25/04, on Ventria's proposal to grow pharmaceutical rice in California in 2004

4) **Seed dispersal through flooding:** Much of southeast Missouri was once swampland. An extensive network of ditches was dredged in the early 20th century to drain the land for agricultural use. Mississippi, Cape Girardeau and Scott Counties – sites of the proposed field trials – border the nation's largest river. The Mississippi is flood-prone, as demonstrated in significant floods in 1993 and 1995 that caused much damage. Pharmaceutical rice carried on floodwaters of the Mississippi or the region's many other waterways could deposit it miles away from the field trial sites.

5) **Scale of planting:** Contamination potential increases dramatically with the size of the planting, and Ventria's planned 200 + acres would be by far the largest pharm crop plantings yet attempted anywhere in the world. The company says it plans to eventually grow 28,000 acres of pharm rice in Missouri, which would dramatically increase the level and scope of contamination.

6) **The human factor:** Human error in transport, processing and disposal represents still another avenue of pharm crop escape.[20] Nearly all Missouri rice is dried, stored and milled by Riceland Foods, a grower-owned cooperative.[21] Riceland has 11 rice receiving and drying facilities in the Bootheel and northeast Arkansas,[22] including one within 20 miles of Sikeston, MO, near the reported site of one or more field trials (see Attachment 2). Two important rice breeding stations – the Missouri Rice Research and Demonstration Farm (Dunklin County) and the Delta Research Center (near Portageville) – are also in the area. This raises the question of whether rice seed stocks might become contaminated with Ventria's pharmaceutical traits. In addition, Riceland Foods operates a large international exporting facility in New Madrid, MO, less than 20 miles from Sikeston.

7) **Is Ventria trustworthy?** Ventria's commitment to strict gene containment practices was thrown into doubt in 2004 by an apparent violation of USDA field trial standards. According to the *Contra Costa Times*, the USDA sent a letter to Ventria citing the company for growing its pharm rice "within 100 feet of rice intended for human and animal food;"[23] 100 feet is the mandatory isolation distance established by USDA for pharmaceutical rice in 2002.[24] In addition, Ventria's recent switch from a two-year to one-year fallow period following cultivation of its pharm rice[25] means a greater likelihood of pharm rice volunteers contaminating a commercial rice or soybean crop grown subsequently on the same field. Finally, according to participants in the California Rice Commission hearings in 2004, a Ventria representative claimed that the company had its federal permits in order for cultivation of its rice in California, when in fact the FDA had not (and still has not) responded to Ventria's petition[26] (see "Regulatory Situation: FDA" below)

Perhaps the most persuasive evidence that Ventria's rice will contaminate commercial rice comes from Ventria itself. In a filing with the FDA, Ventria Vice President Delia Bethell candidly admits "the possibility of the inadvertent introduction of LF164 [one variety of pharm rice] at low, adventitious levels into commercial rice varieties."[27]

Economic Impacts of Adulteration

Missouri and Arkansas authorities and rice growers should give serious consideration to the impact on both domestic and export markets should Ventria's pharmaceutical proteins contaminate rice intended for food or feed. Within the U.S., the FDA's zero tolerance standard

for plant-made pharmaceuticals in food could condemn such rice as adulterated, and hence unsaleable. Even if FDA didn't condemn contaminated rice as adulterated, buyers might well reject it. America's $500 billion food industry demands zero tolerance in no uncertain terms. The National Food Processors Association demands "no use of food or feed crops for plant-made pharmaceutical production without a '100% guarantee' against any contamination."[28] The Grocery Manufacturers of America also demand zero tolerance: "Anything less than 100% containment also will subject all participants in the drug development efforts – from farmers to pharmaceutical companies – to potential liability for bodily injury to consumers..."[29]

The impact on exports could be even more serious. Over 99% of Missouri rice is long-grain, and roughly half of U.S. long-grain rice is exported. Long-grain rice comprises nearly 80% of U.S. rice exports,[30] with the largest markets (2001-2002) being Mexico, Central America, the European Union, Saudi Arabia, Canada, and South Africa.[31] None of these important export markets has approved genetically engineered rice, much less pharmaceutical rice, for importation. Mexico has banned production and importation of pharmaceutical corn,[32] and EU countries are GMO-sensitive. Thus, pharmaceutical contamination of Missouri rice could put a good deal of the state's rice exports at risk.

> *"Anything less than 100% containment also will subject all participants in the drug development efforts – from farmers to pharmaceutical companies – to potential liability for bodily injury to consumers and for economic losses and damage to the brand names of affected businesses."*[29]
>
> Grocery Manufacturers of America, in comments to FDA on biopharming, 2/6/03

The likely response of GMO-sensitive export markets to even low levels of pharmaceuticals in rice can be gauged by their actions in response to contamination of the U.S. corn supply with unapproved StarLink GE corn in 2000. According to the USDA, outstanding sales of U.S. corn to Japan at the end of 2000 were down about 21% from the previous year, and the gap had widened to 44% by mid-April 2001.[33] Even before StarLink, the European Union drastically cut purchases of U.S. corn when ***approved*** varieties of engineered Bt varieties were introduced in 1996.

Finally, the mere presence of pharmaceutical rice in the Missouri / Arkansas region could have market impacts. European grain traders and consumers would likely react as negatively as the Japanese did to the prospect of pharmaceutical rice in California. A letter from the Japanese Rice Retailers Association to the California Rice Commission in April 2004 stated: "We wish to inform you that if you approve Ventria's request [for a 2004 field trial of pharm rice], California's rice market in Japan will be seriously threatened." Joe Carrancho, 40-year rice grower and past president of the Rice Producers of California, agrees: "If the Japanese have the perception -- underline perception -- that our rice has (genetically modified organisms) in it, then we're done. You can put a bullet in our head."[34]

What Rice Growers are Saying

Arkansas rice growers are rightly concerned about pharmaceutical rice being grown so close to their state, especially since most Missouri rice ends up at a Riceland Foods receiving facility in Corning, AR and a mill in Jonesboro, AR.[35] The Arkansas Rice Growers Association recently passed a resolution calling for restrictions on the cultivation of "pharm" crops.[36] The US Rice

Producers Association has also expressed concern about "pharma rice in Missouri."[37]

Regulatory Situation

> *"Company's move here may reflect relaxed climate"*
> St. Louis Post-Dispatch, 12/1/04
>
> *"Biofarming faces few Missouri rules"*
> St. Joseph News-Press, 1/23/05

Ventria's move to Missouri was motivated in part by a laxer regulatory environment than the company faced in California.[38] California authorities required additional safeguards to make up for inadequate federal standards.[39] Even though USDA imposed stricter gene containment measures and beefed up its inspection activities in 2002, such measures can only reduce, not ***prevent***, contamination, and this will simply not suffice for the zero tolerance demands of regulators, the food industry and export markets. As Joe Carrancho (quoted above) said of Ventria's California production protocol: "This is probably the most stringent protocol I've ever seen. And it's not enough."[40]

The bottom line is that federal regulators have a fundamentally contradictory policy. While they properly ban even trace amounts of plant-made pharmaceuticals in food or feed, they allow open-air cultivation of Ventria's crops, virtually ensuring contamination of rice meant for food and feed use.

U.S. Department of Agriculture

Though USDA has authority over Ventria's pharm rice in the field, it did not conduct any tests to determine whether Ventria's pharm traits were spreading to food-grade rice or related weed species in California, nor did it examine the potential for a noxious weed risk from the spread of Ventria's traits. Though USDA has promised to do environmental assessments of the proposed Missouri field trials, past experience suggests they will be superficial and deny the risks of contamination that most scientists acknowledge.

U.S. Food and Drug Administration

As noted above, any food-grade rice contaminated with Ventria's pharmaceuticals could be considered "adulterated," and so subject to seizure and destruction. This was what happened to half-a-million bushels of soybeans contaminated with pharmaceutical corn in Nebraska in 2002. Seeking to avoid a similar debacle, in November 2003 Ventria petitioned the FDA to: 1) have one of its pharmaceutical rice varieties declared "generally recognized as safe" (GRAS) in the event that it contaminates food-grade crops; and 2) grant Ventria permission to put rice residues remaining after extraction of the pharmaceutical (lactoferrin) into the food and feed supply.[41] GRAS designation would help shield Ventria from liability for contamination incidents. But FDA has not responded to Ventria's petition, over a year since it was filed, meaning that pharmaceutical-contaminated rice could still be considered adulterated. But even if FDA does go along, GRAS status would do nothing to prevent the market rejection of contaminated rice discussed above. In addition, GRAS designation by the FDA would not permit the company to sell its rice-grown pharmaceuticals, which would still have to pass a lengthier drug or food additive review process (depending on the application).

> *"The current U.S. regulatory framework does not inspire confidence among our collective members that that these drug and chemical crops will remain isolated and confined and not contaminate the food supply."*[29]
>
> Grocery Manufacturers of America, in comments to FDA on biopharming, 2/6/03

U.S. Environmental Protection Agency
The EPA has not assessed Ventria's pharm rice despite evidence that its pharmaceutical proteins have antibacterial and anti-fungal properties and might lead to creation of hardier weeds, harm non-target organisms, and/or disrupt soil ecology.[42]

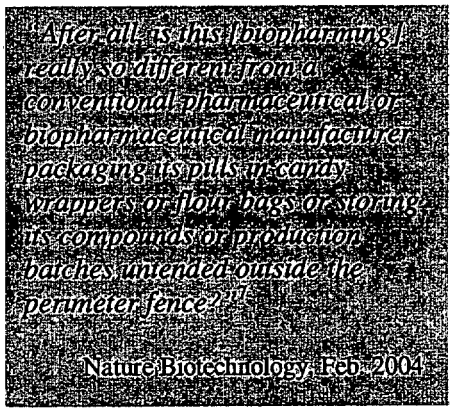
Missouri Department of Agriculture
USDA will submit Ventria's applications to the Missouri Department of Agriculture, which can either sign-off on the field trials, suggest changes to permit conditions, or oppose them. Missouri DoA opposition could stop the field trials, even though ultimate authority resides with USDA.

Arkansas State Officials
Opposition is mounting in nearby Arkansas, where state senator Jerry Taylor recently stated: "We're either going to try to have a ban on it [pharm crops] in Arkansas or at least have a controlled-environment requirement."[43]

Potential Human Health and Environmental Impacts[44]

The USDA has sole authority over pharm crops during field trials. The FDA has not reviewed Ventria's pharm compounds for potential human health impacts, nor has the EPA examined them for possible environmental effects. Yet there are a number of potential hazards that should have been investigated before these crops were ever planted out-of-doors, and certainly should be before any more field trials are allowed.

One of Ventria's lead products is recombinant human lactoferrin, an artificial protein similar to (but different than) the lactoferrin found in human breast milk. While lactoferrin has antimicrobial properties, it paradoxically may exacerbate infections by certain pathogens capable of using it as a source of needed iron. Such pathogens include bacteria that cause gonorrhea and meningitis, as well as the *H. pylori* bacteria implicated in ulcers and certain forms of stomach cancer. According to Dr. Eugene Weinberg, human lactoferrin "might not be a successful therapeutic agent for *H. pylori* and, indeed, could intensify the infection."[45] The possibility of aggravated infections is a potential risk from the contamination of food rice by lactoferrin that argues against growing this rice outdoors.



Ventria's rice-expressed lysozyme and lactoferrin have two characteristics of proteins that cause food allergies: resistance to digestion and to breakdown by heat. Its lactoferrin has a third characteristic, structural similarity to a known food allergen, lactoferrin from cows. These allergenic characteristics may explain why noted food allergist Steve Taylor stated that FDA regulations "will have to be rethought before rice-grown lactoferrin ... can be approved for production."[46]

Pharmaceutical proteins generated by inserting human genes into plants, bacteria or other mammals are usually different than their natural human counterparts. These differences may cause the body to perceive them as foreign, resulting in immune system responses. These

immune reactions can deactivate the pharmaceutical, and in some cases also deactivate the body's natural version of the protein, resulting in autoimmune disorders. Careful study is required to determine whether rice-expressed lactoferrin or lysozyme could cause such potentially dangerous reactions.

Ventria's rice-produced pharmaceuticals have antibacterial and antifungal properties. If these traits are passed to related weed species such as red rice, they could lend these weeds a fitness boost, promoting their spread. These weed species, as well as contaminated food-grade rice that sprouts in subsequent years from unharvested seed, could harbor these pharmaceutical traits and thus serve as a "genetic bridge" to pass the traits back to food-grade rice in the future.

Biopharming: Hype versus Reality[47]

Ventria and its supporters are making big promises. CEO Scott Deeter predicts 28,000 acres of pharm crops in Missouri sometime in the future.[48] Northwest Missouri State President Dean Hubbard dreams of a biotech version of "Silicon Valley" in Maryville, and farmers making "more than twice what they ever dreamed of making in the past."[49] There is even loose talk of 33,000 new Missouri jobs created by the hoped-for biopharmaceutical boom.[50]

"This notion that you lure biotech to your community to save its economy is laughable. This is a bad-idea virus that has swept through governors, mayors and economic development officials."[51]

Economist Joseph Cortright, as quoted in "States, cities court biotech, but is it worth it?," Associated Press, 6/9/04

Claims such as this are founded more on misinformation and wishful thinking than sober judgment, much less a detailed business plan. They recall similar unfulfilled promises made by other biotech companies, and it is virtually certain they will never come true.

The truth is that biotechnology has proven to be extremely risky as an economic development tool. "This notion that you lure biotech to your community to save its economy is laughable," says Joseph Cortright, an economist who co-wrote a report on the subject for the respected Brookings Institution. "This is a bad-idea virus that has swept through governors, mayors and economic development officials."[51] According to Cortright, biotech has become firmly rooted in just three regions – San Francisco, San Diego and Boston – by virtue of their strong venture capital communities, stellar academic institutions and highly-educated workforces (six other major metropolitan areas have established, but less developed biotech industries). Cities (including many much larger than Maryville) without these prerequisites have failed, and will likely continue to fail, despite investing millions of dollars in companies like Ventria.

Cortright is not alone. The Wall Street Journal recently reported that publicly-traded biotechnology companies lost investors an astounding $41 billion from 1990-2003.[52] Comparing biotech firms to 1990's-era "dot.coms," the author notes that: "Some companies survive as long as two decades on investors' largesse without developing a revenue-producing drug." Others who have studied the issue conclude that the "biotech revolution" is largely a myth.[53]

"Some companies survive as long as two decades on investors' largesse without developing a revenue-producing drug."[52]

From: "Biotech's Dismal Bottom Line: More Than $40 Billion in Losses," Wall Street Journal, 5/20/04

Though it has come at a high price, at least biotechnology has produced a handful of successful biopharmaceuticals – but these drugs are all produced in contained and controlled

fermentation facilities using genetically engineered bacteria or animal cell cultures – not plants grown out-of-doors. In fact, not a single "plant-made pharmaceutical" has received FDA approval, despite field trials dating back to 1991. Reasons for the failure of plant-based "biopharming" include technical obstacles unique to the plant system, and lack of control over production conditions in the form of fluctuating environmental conditions.

Lacking products or revenue, it is not surprising that these companies live – and die – on venture capital and government subsidies. As noted above, Ventria's $35 million subsidy package from Missouri includes $5 million to finance operating costs "before profits are made." This recalls Iowa's investment of $6 million in ProdiGene, once a leader in the development of pharmaceutical corn. Iowa's only return on this investment was an expensive contamination incident in 2002.[54] Unable to pay USDA-imposed penalties, the strapped company was taken over by Stine Seed Company.[55] CropTech, once a leading developer of pharmaceutical tobacco, promised to save struggling Virginia tobacco growers, but went bankrupt after receiving $12 million in government subsidies over its 10-year history – without producing a single product.[56] Epicyte Pharmaceutical, another pharm corn prodigy, went bankrupt in 2004, its assets bought up by Biolex of North Carolina. In a clear sign of the times, Biolex is abandoning Epicyte's outdoor production of pharmaceuticals in corn to focus on non-food plants grown in contained and controlled facilities.[57]

> *"Farmers' salvation does not lie at the bottom of a test tube. What they need most of all are sensible farm policies."*[61]
>
> Editorial, The Economist, March 25-31, 2000

Where commercial products and revenue are lacking, there is no shortage of hype and dreams. But whether it is ProdiGene predicting millions of acres of pharm corn by 2010,[58] or Ventria and its backers promising to turn Maryville into biotech's "Silicon Valley" and make Missouri rice farmers rich, it does not serve the public interest to make exaggerated claims and raise false hopes concerning this unproven technique. It is interesting to note that the established ag biotech firms are leery of biopharming; in fact, industry leader Monsanto shut down its pharm crop division in 2003 due to "uncertainty of the longer-term reward from a highly capital-intensive business."[59]

Conclusion

Pharmaceutical food crops are an idea whose time is past. They will almost certainly contaminate conventional crops, spelling trouble for growers. They are strongly opposed by consumers and the food processing industry, who want nothing less than drug-laced food products. They could easily cause loss of exports to GMO-sensitive markets in the European Union, Mexico and elsewhere. Economists tell us they are an extremely high-risk investment that is likely to fail. Federal regulators have not reviewed them for potential human health or environmental impacts. And they simply aren't working.

Genetically engineered pharmaceutical crops represent the cutting edge of a highly-industrialized agricultural system that has mowed down countless family farmers. Indeed, the number of farms in southeast Missouri has declined dramatically over the past decades, and Missouri rice growers now number just 250 to 350, despite steadily increasing production figures.[60] Overall, the U.S. now has the unenviable distinction of being perhaps the only nation in the history of the world with more people in jail than on the land growing food. As *The*

Economist put it in an editorial on hi-tech farming: "Farmers' salvation does not lie at the bottom of a test tube. What they need most of all are sensible farm policies."[61]

This is a huge and complex problem, but several things are clear. Farmers need production systems that generate products fetching a decent price, and marketing systems that allow them to capture a fair share of that price. And consumers are increasingly demanding high-quality, locally-grown foods. Initiatives in Missouri that are bringing farmers and consumers together to build a healthy food system include the Food Circles Networking Project, and the Missouri Rural Crisis Center, which seeks to preserve family farming through such projects as the Patchwork Family Farms, a cooperative of Missouri hog farmers who raise and market high-quality pork in the state. State and federal officials should be supporting worthy initiatives of this sort rather than fund risky, expensive, hi-tech ventures like Ventria's pharmaceutical rice.

Pharmaceutical Rice in California

Introduction

Since 1997, Ventria Bioscience or its predecessor Applied Phytologics has been conducting outdoor field trials of rice varieties genetically engineered to produce pharmaceutical proteins in California's rice-growing Central Valley. These proteins, which are generated in and extracted from grains of rice, include artificial versions of human lactoferrin, lysozyme and alpha-1-antitrypsin. Last year, Ventria was authorized by the U.S. Department of Agriculture (USDA) to grow 93 acres of pharmaceutical rice in the Central Valley. This year, the company's bid to grow 120 acres of pharm rice in Southern California was rejected; on May 13th, Ventria was granted permission to grow one acre in the Central Valley under permit # 03-365-01R.[62]

While over 100 biotech pharmaceutical proteins produced in *contained* and *controlled* fermentation facilities have been approved by the FDA and are already helping people in need, pharmaceutical crops such as Ventria's rice represent an ***experimental*** and ***unproven*** application of biotechnology. Not a single "plant-made pharmaceutical" (PMP) has been approved by the U.S. Food and Drug Administration (FDA), despite several clinical trials, numerous industry promises, and field trials dating back to 1991. Federal regulators frankly admit that they are treading new ground. The comment of the FDA's Michael Brennan at a conference on PMPs four years ago is still applicable today:

> *"And I think to be honest, the FDA is used to applying regulations to manufacturing plants, but not to plants used for manufacturing. So a lot of this is new to us as well, and that's why I won't be able to answer any questions at the end!"*[63]

As we discuss below, contamination of conventional rice by Ventria's pharm rice appears inevitable. In this report, we detail a number of serious concerns and unanswered questions regarding the potential human health, environmental and economic impacts of Ventria's pharmaceutical rice. These concerns have not been adequately addressed by the U.S. Dept. of Agriculture (USDA), the Environmental Protection Agency (EPA), or the FDA. Therefore, we call on the California Department of Food and Agriculture (CDFA), the California Department of Health Services (CDHS), and the California Environmental Protection Agency (Cal-EPA) to conduct a thorough review of Ventria's pharmaceutical-producing rice to address these concerns. CDFA should examine the likely economic effects on California rice growers should Ventria's traits be discovered in food-grade rice. CDHS's Division of Environmental and Occupational Disease Control and Division of Food, Drug and Radiation Safety could address the potential human health impacts of Ventria's pharmaceutical traits as contaminants in food rice, while Cal-EPA should carefully review the possible environmental impacts of these pharm crops.

Because of the potential risks and the great scientific uncertainty surrounding this unproven application of biotechnology, we believe a prudent approach is called for to protect the interests of California consumers and farmers. Thus, we further urge California authorities to consider a moratorium on the cultivation of Ventria's pharmaceutical rice and other pharm crops.

Contamination is Inevitable

There is a fundamental contradiction in the federal government's policy on pharmaceutical-producing food crops. While the government properly maintains a zero-tolerance standard for contamination of food with plant-made pharmaceuticals, it nevertheless permits them to be grown outdoors in the direct vicinity of food-grade crops of the same species, posing a high risk of contamination.

The zero tolerance standard currently in force for pharm crop residues in food or feed is unlikely to be changed because zero tolerance is strongly supported by the powerful food industry. The National Food Processors Association demands "no use of food or feed crops for plant-made pharmaceutical production without a '100% guarantee' against any contamination."[64] The Grocery Manufacturers of America also demand zero tolerance: "Anything less than 100% containment also will subject all participants in the drug development efforts – from farmers to pharmaceutical companies – to potential liability for bodily injury to consumers..."[65]

But is 100% containment of food crops engineered to produce drugs likely or even possible? Numerous authorities have made it perfectly clear that it is not. Two committees of the National Academy of Sciences have warned of the high risk that pharmaceuticals from pharm crops will contaminate the food supply.[66] Leading agronomists such as Dirk Maier of Purdue University have made the same point.[67] A leading journal in the field, *Nature Biotechnology*, has published two editorials on this theme,[68] asking whether pharm crops are "...really so different from a conventional pharmaceutical or biopharmaceutical manufacturer packaging its pills in candy wrappers or flour bags or storing its compounds or production batches untended outside the perimeter fence?"

Contamination of human foods with plant-made pharmaceuticals can occur through dispersal of seed or pollen. Wildlife, especially waterfowl, can transport seed for long distances, as can extreme weather events such as floods or tornadoes. Harvesting equipment can carry seed residues to conventional fields, seeds can be spilled from trucks, or unharvested seeds can sprout as volunteers amid the following year's crop. Cross-pollination occurs at considerable distances in high winds or by insect, even with self-pollinating crops such as rice.

That these are more than theoretical concerns is abundantly demonstrated by a growing list of transgene contamination incidents in other crops. In early 2000, at a USDA/FDA-sponsored meeting on pharm crops, Chris Webster of Pfizer stated that: "We've seen it on the vaccine side where modified live seeds have wandered off and have appeared in other products."[69] In the same year, StarLink corn, approved only for use as animal feed, was found contaminating the entire food chain, from processed foods to grain to seed stocks.[70] Despite massive efforts to eliminate it, residues of StarLink continue to be found in the corn supply even today, over 3 years later.[71] In 2001, a variety of GE canola unapproved for sale to Canada's major export markets was found in commercial canola, leading to recalls of thousands of bags of seed and the

incineration of some 10,000 acres of the unapproved GE canola variety. In 2002, ProdiGene, Inc. allowed its pharmaceutical corn to contaminate half a million bushels of soybeans in Nebraska and 155 acres of corn in Iowa.[72] The adulterated soybeans and corn had to be destroyed. In 2003, wheat grown in the U.S. was found to be contaminated with biotech crops. Supposedly conventional tomato seeds were unwittingly sent around the globe for seven years until transgene contamination was detected in late 2003, ironically, by UC Davis researcher Nicholas Ewing, who was conducting research on pharmaceutical-producing tomatoes.[73] The Union of Concerned Scientists recently demonstrated widespread low-level contamination of conventional corn, soy and canola ***certified seed*** stocks with commercialized transgenic traits.[74] These findings were anticipated by authorities like Walter Fehr, Director of the Office of Biotechnology at Iowa State University, who was quoted as saying that transgenic contamination of even ***breeder*** seed stocks of corn and soy "happens routinely."[75] When certified and even breeder seeds, whose cultivation is subject to extraordinary gene confinement measures, become contaminated, it becomes impossible to believe in 100% containment of pharm genes, no matter how stringent the gene confinement measures that are applied (including geographic isolation).[76]

Ventria's Rice Protocol Will Not Prevent Contamination

Nothing in Ventria's draft protocol for cultivation suggests that it will achieve 100% containment of its pharmaceutical rice. According to the Sacramento Bee, Ventria's protocol:

> *"...is light on some details, including how Ventria will prevent birds from spreading its rice; what constitutes "proper" disposal of rice plants; and whether the company will notify nearby growers."*[77]

The lack of detailed plans to prevent birds from spreading the pharm rice is particularly disturbing. California's Central Valley is one of the most important wintering areas for waterfowl in North America. Viable seed are known to pass through the gut of many waterfowl species, making waterfowl effective dispersal agents for many wetland plant species, including rice.[78] The same study also found that mallard ducks could transport viable seeds for up to 1400 kilometers, about 870 miles.

Ventria's draft protocol also does not deal with the possibility of seed dispersal through flooding. Ventria has grown its rice in the Central Valley, for instance in Sutter County (2001)[79] and Butte County (1997).[80] Historical records show that floods of various magnitude occur not infrequently in the Sacramento Valley.[81] Such flooding would carry pharm rice an indeterminate distance from its original field.

Ventria's recent switch from a two-year to one-year fallow period following cultivation of its pharm rice[82] means a greater likelihood of pharm rice volunteers contaminating a commercial rice crop grown subsequently on the same field.

The 100-foot isolation distance from food-grade rice stipulated in the permit conditions for cultivation of Ventria's rice may not be adequate to prevent cross-pollination. Rice pollen may be able to move up to 360 feet from its source in 22 mph winds, and it has been shown to travel at least 126 feet in 5.6 mph winds. Wind speeds in the Sacramento Valley often exceed 22 mph, and could result in even greater

pollen dispersal.[83] Bees can also cross-pollinate rice plants, and rice breeders have observed that the out-crossing rate increases in the presence of honeybees.[84]

Finally, one press report suggests that Ventria may have violated its 2003 USDA permit by growing its pharm rice "within 100 feet of rice intended for human and animal food."[85] USDA established this mandatory isolation distance for plantings of pharmaceutical rice in 2002,[86] and has confirmed that it applies to Ventria's 2004 pharm rice trial.[87] If this report is true, it casts further doubt ***on Ventria's ability to keep its pharm rice from contaminating food-grade rice.***

In light of the expert testimony and history of contamination cited above, and given the deficiencies in Ventria's draft protocol, we should assume that contamination of food-grade rice with Ventria's pharmaceutical rice, either through cross-pollination, inadvertent seed movement, or human error, is inevitable.

Potential Human Health Impacts

If contamination is inevitable, then the potential human health impacts of exposure to Ventria's pharmaceutical proteins becomes a serious question. While some might argue that consumers' exposure to these proteins from contaminated rice would be at levels too low to be of concern, there is little basis for such assertions. First, the federal government does not monitor for contamination, so any statements about the level of actual or potential contamination of food are speculative, not science-based. Secondly, Ventria is generating extremely high levels of its pharmaceutical proteins in rice, up to 1% by weight of the rice grain, equivalent to 40-50% of the grain's soluble protein.[88] Food rice contaminated with Ventria's pharmaceutical genes could generate equally high levels. Finally, there has not been adequate study to determine what levels of these proteins might cause human health impacts, though we do know that allergies and immune system disorders (see below) in general can be triggered by extremely low levels of immunogenic compounds.

Lactoferrin Inhibits But May Also Promote Certain Pathogens

Lactoferrin is found in bodily secretions, such as breast milk, tears, saliva, gastrointestinal and seminal fluid, as well as in the mucous membranes lining the nose, vagina and lungs. These are the body's portals to the outside world, and hence the entry points for many pathogens. Lactoferrin is also an important component of infection-fighting white blood cells known as polymorphonuclear neutrophils, which circulate in the bloodstream. Accordingly, one of lactoferrin's chief roles is to fight microbial infection. The main weapon in lactoferrin's pathogen-fighting arsenal is its ability to bind free iron at infection sites. Iron is an essential nutrient, for microbes as for humans. Lactoferrin locks up iron, making it unavailable, and thus literally starves many microbial invaders.[89]

Several of Ventria's proposed uses for rice-derived lactoferrin are based on this antimicrobial property, including treatments for bacterial-induced diarrhea and topical infections, as well as (partial) replacement of antibiotics in poultry feed.

As so often in nature, however, closer examination reveals a more complex state of affairs. Microbes have developed several mechanisms to reclaim the iron they require for growth. Some compete with lactoferrin by secreting their own iron-binding compounds (called siderophores)

that then provide them with the iron. Other pathogens have learned the trick of extracting iron directly from lactoferrin and its close relative transferrin – they actually feed on the weapon developed by the body to kill them. Pathogenic bacteria in this latter class include *Helicobacter pyloris* (ulcers and stomach cancers), *Haemophilus influenza* (meningitis), *Bordetella pertussis* (whooping cough), *Legionella pneumophila* (legionnaires' disease), and two species of the genus *Neisseria* that cause gonorrhea and meningitis.[90] *Trichomonas vaginalis*, a protozoan responsible for genital disease in both women and men, can also extract iron from lactoferrin.

According to Dr. Eugene Weinberg, therapeutic use of human lactoferrin could stimulate growth of such pathogens, resulting in an "adverse response." Weinberg notes that human lactoferrin "might not be a successful therapeutic agent for H. pylori and, indeed, could intensify the infection." The gut bacterium H. pylori is implicated in causing ulcers, chronic gastritis and certain forms of stomach cancer. Thus, if food rice were to be contaminated with Ventria's lactoferrin, consumers of this rice who happened to have H. pylori infections in their guts could find those infections, and their associated conditions, exacerbated. This potential risk deserves careful evaluation. While Weinberg believes that human lactoferrin (Lf) has therapeutic potential, he argues that "[p]recaution is needed ... to avoid ... introduction of the protein [lactoferrin] to tissues that may be infected with specific protozoa or bacteria that utilize Lf in their acquisition of host iron."[91]

Rice-derived recombinant human lactoferrin and lysozyme are not identical to native human lactoferrin and lysozyme

Ventria and its collaborators often refer to its recombinant proteins as if they were the human milk proteins lactoferrin and lysozyme. Such a characterization is incorrect.

In general, recombinant proteins may differ from their native counterparts in two major ways: 1) Amino acid sequence, as encoded by the transgene; 2) Post-translational processing, a function of the host organism. One form of post-translational processing is glycosylation, or attachment of carbohydrate groups to the surface of the protein. Animals and plants attach different types of carbohydrate groups to proteins. It has been demonstrated that recombinant, rice-expressed human lactoferrin and alpha-1-antitrypsin are glycosylated differently than their native counterparts in humans.[92] The author notes that the latter difference may affect the recombinant proteins' stability. Any increase in stability would be a warning flag, as digestive stability is a characteristic of food allergens (see "Allergenicity" below).

The recombinant versions might also have different primary amino acid sequences than the native human proteins because genetic engineering sometimes results in integration of a fragmented or otherwise disrupted transgene into the plant's genome (i.e. total genetic material).[93] In studies listed on Ventria's website, company scientists compared only the N-terminal sequences of the human and recombinant proteins rather than the full sequences.[94] In the case of lysozyme, a 130 amino acid protein, only 11 amino acids at the N-terminals of the native and recombinant versions were sequenced and compared; 10 of 11 of these amino acids were demonstrated to be identical. The identity of the other 119 amino acids was apparently not determined, thus only 8% of the amino acids of the two proteins were demonstrated to be identical. A Scientific Advisory Panel to the U.S. Environmental Protection Agency (EPA) has

recommended full-length amino acid sequencing of plant-produced recombinant proteins, as one or two point mutations can affect the protein's allergenicity or other properties. Overall, the equivalence testing conducted by Ventria scientists and it collaborators in studies listed on Ventria's website and in its comprehensive patent[95] does not meet standards established by this EPA Scientific Advisory Panel in a similar context.[96] There is no mention of tertiary structure comparisons, so the recombinant and natural versions could differ in conformation as well.

Thus, researchers have found clear differences in glycosylation between human lactoferrin and its rice-expressed counterpart that could cause the latter to differ from the former in stability or allergenic properties. Rice-expressed lysozyme and lactoferrin may differ in amino acid sequence from their native human counterparts. The extent of the differences, as well as the potential human health implications, should be examined.

Immune System Disorders

The mammalian immune system serves to protect the body from micro-organisms, viruses, and substances recognized as foreign and potentially harmful to the body. The immune system works by recognizing and responding to large molecules (usually proteins) called antigens. Any substance or organism that contains such antigens is recognized and attacked by the immune system. Proteins that the body recognizes as "self" (e.g. insulin) are normally not attacked. Immune system disorders occur when the immune response is excessive, inappropriate, or lacking. Allergies occur when the immune system overreacts to a substance that, in the majority of people, the body perceives as harmless (such as a food protein). Autoimmune disorders occur when the immune system responds to certain of the body's own proteins as if they were antigens, thus destroying or damaging normal body tissue. The studies discussed below raise questions about the potential for recombinant, rice-derived lactoferrin and lysozyme to lead to an allergic response and/or autoimmune disorder.

Allergenicity

Any novel transgenic protein bears close scrutiny as a potential allergen. According to Bo Lönnerdal, a scientist at the University of California, Davis, recombinant, rice-expressed lactoferrin and lysozyme are stable to digestion and heat,[97] two properties widely regarded as characteristic of food allergens.[98] Digestive stability is particularly pronounced in infants, whose guts secrete less pepsin and are less acidic (pH 4-5) than adult guts (pH 1-3). A material safety data sheet on rice-expressed human lysozyme states that: "Prolonged or repeated exposure may cause allergic reactions in certain sensitive individuals."[99] Ventria's lactoferrin has a third characteristic of food allergens: significant amino acid sequence homology to a known human allergen, bovine lactoferrin, an allergen found in cow's milk.[100] These allergenic properties may explain why noted food allergist Steve Taylor stated that FDA regulations "will have to be rethought before rice-grown lactoferrin, and other human proteins made by genetically modified organisms, can be approved for production..."[101]

Autoimmune Disorders

Two lines of evidence – one general and one specific to lactoferrin – suggest that Ventria's proteins may have the capacity to cause immune system dysfunction.

First, there is a growing body of evidence demonstrating puzzling, unexpected and in some cases dangerous immunologic responses to biopharmaceuticals produced in genetically engineered cell cultures.[102] In these cell culture production systems, a human gene encoding a medically useful protein such as insulin is spliced into bacteria or mammalian cells, which then produce a recombinant version of the protein, known as a biopharmaceutical. While the immune system does not normally attack a bodily protein because it is recognized as "self," it may respond to the corresponding biopharmaceutical due to subtle differences that cause the body to recognize it as foreign. The precise nature of these differences has not been established in most cases and is a subject of intense research; they could involve differences in post-translational processing, tertiary structure, and/or primary amino acid sequence.

In some cases, the administered biopharmaceutical merely elicits an immune system response that reduces or eliminates the drug's potency. This phenomenon has been observed in some patients receiving recombinant blood clotting Factor VIII and the multiple sclerosis drug beta-interferon. In other cases, the immune system detects that the engineered drug is different (i.e. treats it as foreign), yet the antibodies produced against the engineered drug also target the natural counterpart, thereby leading to potentially disastrous consequences. For instance, a recombinant version of megakaryocyte growth and development factor (MGDF) produced by Amgen was discontinued in clinical trials because some patients receiving the drug mounted an immune attack on both Amgen's recombinant MGDF and their own natural version of MGDF, resulting in bleeding. A similar phenomenon might be responsible for up to 160 cases of red cell aplasia (virtual shutdown of red blood cell production) observed in patients treated with recombinant erythropoietin, a hormone that stimulates red blood cell production. The important fact to keep in mind here is that these reactions to recombinant biopharmaceuticals have taken biotech companies and regulators alike ***by surprise***. Dr. Burt Adelman, head of research & development at Biogen, found the immune reactions to MGDF "stunning."

> *"The conventional wisdom had been that this was a theoretical risk ... nobody saw it coming. If you're in my business, it's really unnerving."*[103]

In other words, although the natural human protein and the corresponding engineered biopharmaceutical appear to be identical, the immune system is able to detect a difference that scientists, at present, cannot. The FDA has implicitly recognized this fact. At a meeting in 2002 about human plasma-derived drugs, the FDA's Chris Joneckis noted that:

> *"Despite best efforts to detect product differences and predict the impact of manufacturing changes, these surprises do continue to occur."*[104]

If tightly-controlled fermentation production of mammalian cell-produced "human" drugs is causing such stunning, unpredicted and in some cases hazardous immune reactions, what are we to think of plant-produced pharmaceuticals such as lactoferrin produced in plants subject to the

"manufacturing changes" imposed by nature in the form of widely varying microclimates and microhabitats, insect infestation, etc.?

A second, admittedly more speculative, immunologic concern specific to rice-expressed lactoferrin is suggested by the unexplained presence of anti-lactoferrin antibodies in the blood stream of many patients suffering from a wide range of autoimmune disorders:

> *"...anti-LF autoantibodies are found in several autoimmune conditions, including rheumatoid vasculitis, rheumatoid arthritis, systemic lupus erythematosus, ulcerative colitis, primary sclerosing cholangitis and Crohn's disease."*[105]

While these anti-lactoferrin antibodies have not yet been demonstrated to have pathophysiological significance, they have been shown to be correlated with markers of disease activity in patients with rheumatoid arthritis and systemic lupus erythematosus.[106] One report suggests that when anti-lactoferrin antibodies of the IgG class bind to lactoferrin in the synovial fluid of rheumatoid arthritis sufferers, they cause lactoferrin to release iron, which in its unbound state is implicated in arthritic inflammation and tissue damage.[107] One team recommends that:

> *"Future research should address the pathophysiological role of anti-lactoferrin ANCA [antineutrophil cytoplasmic autoantibodies] and the influence of anti-lactoferrin ANCA binding on the functional properties of the lactoferrin molecule."*[108]

Lactoferrin expert Dr. Eugene Weinberg agrees: "... an important potential hazard of therapeutic use of hLf [human lactoferrin] in human patients is possible induction of an antibody response."[109] In short, there is great uncertainty concerning a possible pathophysiological role for anti-lactoferrin autoantibodies in autoimmune diseases. Could introduction to the diet of a rice-expressed "human" lactoferrin with subtle but clear differences to the native protein, and with demonstrated resistance to degradation in the gut, elicit potentially hazardous autoimmune reactions? We don't know, but the appropriate research should be undertaken to answer such questions.

Amyloidosis and Mutant Proteins

Hereditary systemic amyloidosis is a rare disease characterized by the deposition of insoluble protein fibers (called amyloid fibrils) in various organs and tissues. The amyloid fibrils result from mutant forms of certain cellular proteins. These mutations cause the cellular proteins to change their three-dimensional shape and become flatter (so-called beta-sheet structure), allowing them to stack up together like sheets of paper to form a fiber which becomes insoluble. Over time, the amyloid fibrils build up in various organs and tissues, making then stiff and reducing their ability to function. One rare form of the disease caused by mutant lysozyme usually presents in middle age and is marked primarily by "slowly progressive renal impairment that can take decades to reach end-stage."[110] The three known mutant versions result from three point mutations in lysozyme: threonine for isoleucine at position 56, arginine for tryptophan at position 64, or histidine for aspartic acid at position 67. In each case, the mutant lysozyme auto-aggregates to form fibrils with a characteristic beta-sheet structure.

As noted above, Ventria reports sequencing only the 11 amino acids at the N-terminal of recombinant lysozyme; the identities of the amino acids at positions 56, 64 and 67 were not determined. Ventria scientists did demonstrate that their rice-expressed lysozyme has antimicrobial activity, which presumably is dependent on the protein molecule assuming its proper three-dimensional conformation, which in turn argues against the conformation-changing point mutations discussed above. Yet circumstantial evidence is not adequate. Ventria should follow the advice of numerous expert bodies and fully sequence recombinant lysozyme to detect these or any other potentially hazardous mutations resulting from its production in rice.

FDA Fails to Consider Unintended Exposure

The inevitable contamination of food-grade rice with Ventria's recombinant proteins raises the question of unintended exposure, which is not even considered by our federal regulators. The FDA plays virtually no role in pharm crop regulation unless a company, often after 5-10 years of outdoor field trials, reaches the clinical trial stage. To the limited extent that FDA may exercise authority in the field, its oversight will be focused on preventing contamination of the pharm crop, not on preventing pharm crop contamination of the food supply.[111] Because the Central Valley is a major rice-growing region, widespread contamination of food-grade rice and exposure of some people to at least low levels of the experimental pharmaceuticals is entirely conceivable, especially given the gene containment lapses described above. Of course, allergy-prone infants and young children as well as adults could be exposed unintentionally through food contamination. One would think that the issue of unintended exposure of the population to untested, potentially hazardous novel proteins would have been dealt with by now, over three years after StarLink corn massively contaminated the food supply and potentially caused food allergies in the exposed population, but such is not the case.[112]

Potential Environmental Impacts

Ventria's pharmaceutical rice varieties could also have negative environmental impacts, such as creation of hardier weeds, damage to non-target insects, and/or disruption of soil ecology. Experimental cultivation of Ventria's pharmaceutical rice in the Central Valley since 1997 has provided the opportunity for recurrent gene flow to two related weed species as well as to food-grade rice. Wild rice (*Oryza rufipogon*) is a federally listed noxious weed that has been found in California in the past, and gene flow between cultivated rice (*Oryza sativa*) and wild rice is well known.[113] Annual red rice (also *O. sativa*) was recently identified in the northern Central Valley's Glenn County, by some accounts in a field grown from certified seed, and is considered a "serious risk to the California rice industry."[114] The increasing scale of Ventria's field trials – from 6 acres in 1999 (the first year for which acreage is reported) to 93 acres in 2003 – increases the risk of gene flow and effectively reduces the level of governmental oversight. We are aware of no testing to determine whether gene flow has occurred into weed species, but believe such monitoring should be required, with the results made publicly available.

Most importantly, Ventria's pharmaceutical traits may confer a fitness boost on contaminated cultivars or weeds, creating or exacerbating a noxious weed risk. Three of these substances – lysozyme, lactoferrin and alpha-1-antitrypsin – have antibacterial, antifungal and/or insecticidal

properties. Recombinant human lysozyme expressed in transgenic tobacco[115] has been shown to confer enhanced resistance to the fungus *Erysiphe cichoracearum* and the phytopathogenic bacterium *Pseudomonas syringae pv. tabaci.* Carrots transformed to express recombinant human lysozyme exhibit enhanced resistance to the carrot pathogens *Erysiphe heraclei*, a fungus causing powdery mildew, and *Alternaria dauci*, a pathogen causing leaf blight.[116] Alpha-1-antitrypsin is a serine protease inhibitor, a class of compounds being tested in many plants as plant insecticides, and some members of which also cause pancreatic damage in animals upon medium-term oral exposure.[117] It is possible that these same proteins will lend rice resistance to similar rice pathogens or insect pests. If these traits are transferred to red rice or wild rice, they may confer a fitness boost to these weedy species, enhancing their survival and making these already noxious weeds still more difficult to control. Crossing with cultivated rice would likely create hardier volunteers, which could also become more difficult to control. These hardier weeds or volunteers could then serve as a genetic bridge or reservoir to transfer the traits back to cultivated rice.[118]

Transgenic proteins can also "leak" from plant roots in a process called rhizosecretion, even when the plant has not been engineered for this purpose. The Bt protein found in most Bt corn varieties has been shown to rhizosecrete into soil and survive in active form adhering to soil particles for at least 180 days.[119] Such rhizosecreted proteins may have significant impacts on soil microbiota. Lysozyme, which as noted above has been experimentally engineered into carrots and tobacco, has also been introduced into potatoes. Lysozyme-containing root exudates of potatoes engineered with the T4 lysozyme gene have been shown to kill 1.5 to 3.5 times as many bacteria (*B. subtilis* as indicator species) as the root exudates of a control line.[120] Rhizosecretion may not be an issue with Ventria's pharmaceutical rice, because the seed-preferred promoters used by the company direct most or all of the transgenic protein to the rice endosperm rather than the roots or other tissues.[121] Still, it would be advisable to analyze the rhizosphere (root-associated soil) of pharm rice to rule out the presence of Ventria's transgenic proteins as well as to detect any adverse impacts on soil microbiota due to unintended effects of the transformation process.

Despite these potential environmental risks, the EPA is not involved in the regulation of Ventria's pharm crops. This might seem surprising given that the EPA is the federal agency responsible for genetically engineered "plant-incorporated protectants," a category that includes antifungal, antibacterial and antiviral agents as well as insecticidal compounds, and considering that Ventria's pharmaceutical proteins possess one or more of these properties. There are two reasons that Ventria can bypass EPA regulation: 1) GE plants are regulated according to the ***intended use*** rather than the ***intrinsic properties*** of their transgenic proteins, and Ventria has not indicated that it intends its rice to be pesticidal; and 2) Even when the intended use ***is*** pesticidal, EPA regulation is triggered only by field trials of over 10 acres. The USDA has also failed to conduct any environmental assessment of Ventria's lactoferrin- and lysozyme-producing rice varieties. Thus, Ventria's two major pharm crops have not been subjected to any review for environmental impacts in the seven years of their cultivation in California.

Deficiencies in the Federal Regulatory System

The discussion above makes it clear that the State of California cannot rely on the federal government to ensure that the State's consumers, farmers and environment are protected from potential harm by Ventria's experimental plantings of pharm rice in California. Loopholes in federal regulation, many of which were pointed out by a National Academy of Sciences' committee two years ago,[122] can be summarized as follows:

1) Despite a proper zero tolerance standard for Ventria's plant-made pharmaceuticals in food and feed, USDA and FDA allow open-air cultivation of these crops in a rice-growing region, which will almost inevitably result in pharmaceuticals adulterating the food and feed supply.

2) The FDA does not regulate Ventria's pharm rice at the field trial stage, and will not regulate it at any stage if the intended use of the rice is production of a research chemical, a medical food,[123] or for export. Although FDA may ultimately review lactoferrin and/or lysozyme produced from Ventria's pharm rice, it will not consider the potential human health impacts of exposure to these pharmaceuticals as contaminants in the food supply.

3) The EPA has not reviewed Ventria's pharmaceutical rice despite evidence that its pharmaceutical proteins possess pesticidal properties and could harm beneficial organisms, create more aggressive weeds, or disrupt soil ecology, because the intended uses of these proteins are not pesticidal.

4) The USDA has not done a single environmental assessment of lysozyme- or lactoferrin-producing rice field trials, despite the potential for a noxious weed risk from transfer of these traits to related cultivars or weed species.

Regulatory Confusion: Pharmaceutical, Food Additive or Food?

We have referred to Ventria's rice as "pharmaceutical rice" for several reasons: 1) USDA field trial permits for this rice granted to Ventria's predecessor, Applied Phytologics, in the period from 1997 – 2001 listed the "pharmaceutical protein produced" phenotype; 2) The permit conditions stipulated by USDA for Ventria's field trials (e.g. 100-foot isolation distance) are those for rice engineered to produce pharmaceuticals and industrial chemicals, not those for field trials of other GE rice varieties; 3) Lactoferrin and lysozyme possess antimicrobial properties; and 4) Several of Ventria's proposed uses for its recombinant proteins – for instance, as additives to oral rehydration formula for treatment of severe diarrhea,[124] or as "topical treatment for bacterial infections"[125] – are explicitly medical in nature.

However, it now appears that Ventria's pharmaceutical proteins may have been reclassified as something other than pharmaceuticals. Consider the following facts. First, for the 2003 and 2004 field trials, USDA changed its designation of Ventria's products from "pharmaceutical proteins produced" to "value added protein for human consumption."[126] Secondly, Ventria reportedly initiated a voluntary consultation on its rice with the FDA in November 2003.[127] The voluntary consultation process is used for GE crops intended for general food use, and it falls far

short of FDA's mandatory pharmaceutical review process. Finally, Ventria representatives have told the California Rice Commission that the company is seeking GRAS (Generally Recognized As Safe) status for its recombinant human lactoferrin and lysozyme from FDA.[128] GRAS status ***exempts*** a food additive from the food additive review process, which is similar in stringency to the FDA's pharmaceutical review process. When contacted, FDA officials refused to comment on how Ventria's rice and recombinant proteins are being regulated – as pharmaceuticals, food additives, GRAS food additives, GE food or otherwise.

This apparent attempt to reclassify Ventria's products from pharmaceuticals to "value-added proteins" is troubling. Ventria's recombinant proteins have pharmaceutical properties, proposed pharmaceutical uses, and they were once classified accordingly by USDA. As detailed above, they pose a number of potential health risks that have not been adequately investigated. In the interests of public health, they should be stringently regulated as pharmaceuticals. Anything less is unacceptable.

Finally, there is evidence that Ventria has already commercialized its rice-expressed lysozyme as a research chemical. The chemical supply house Sigma-Aldrich Inc. currently offers for sale "Lysozyme from human milk, recombinant, expressed in Rice min. 100,000 units/mg protein," as product number L1667 (see www.sigmaaldrich.com for details). Sigma-Aldrich does not state the source of this product, but it is likely to be Ventria, given that Sigma-Aldrich is cited as a collaborator on Ventria's website (see www.ventriabio.com/collaborators/).

Sigma-Aldrich sells this lysozyme "[f]or R&D use only. Not for drug, household or other use."[129] Nevertheless, commercialization of an experimental GE plant-produced compound with pharmaceutical properties is troubling. According to the National Academy of Sciences, such commercialization provides additional incentive for large-scale plantings that increase the likelihood of gene containment lapses,[130] and hence food contamination. Another concern is conflict of interest. USDA oversight of GE crop field trials depends to a great extent on company reports filed with the USDA at the end of the trial, or annually for multi-year permits. Such reports are to include any adverse impacts of the experimental crop. Because self-reporting of adverse impacts to the USDA could entail revocation or non-renewal of the permit, and thus loss of profits, the company's duty to report such adverse effects is clearly in conflict with its financial interest.

Potential Economic Impacts

California authorities should also give serious consideration to the impact on both domestic and export markets should Ventria's pharmaceutical proteins be discovered in rice intended for food or feed. Within the U.S., the FDA's zero tolerance standard for plant-made pharmaceuticals in food would condemn such rice as adulterated, and hence unsaleable. The impact on exports would be even more serious. Approximately one-third of the rice produced in California is exported, primarily to countries that have restrictions on GE foods. In 2002, almost 65% of California's rice exports went to Japan, Taiwan and Korea, all of which require labeling and certification of GE foods. Another 15% of California's rice was exported to Turkey, which is poised to join the European Union and so will have to comply with the EU's strict laws

governing GE food importation.[131] None of these important export markets has approved GE rice, much less pharmaceutical rice, for importation. Thus, pharmaceutical contamination of California rice could put at least 80% of the state's rice exports at risk.

The likely response of these GE-sensitive export markets to even low levels of pharmaceuticals in rice can be gauged by their actions in response to contamination of the U.S. corn supply with unapproved StarLink GE corn in 2000. According to the USDA, outstanding sales of U.S. corn to Japan at the end of 2000 were down about 21% from the previous year, and the gap had widened to 44% by mid-April 2001.[132] Japan turned to Brazil, Argentina, China and South Africa to make up the difference.[133] Corn exports to South Korea also experienced a decline.[134]

Japanese consumers have already voiced their concerns. A letter from Consumers Union Japan to the California Rice Commission dated March 27, 2004 stated: "We wish to inform you that if you approve Ventria's request, California's rice market in Japan will be seriously threatened."

Conclusion

As demonstrated above, there are many serious deficiencies in federal regulation of Ventria's pharmaceutical crops. These deficiencies expose California's consumers, farmers, and rice industry to potential human health, environmental and economic risks. It should also be kept in mind that the manner in which Ventria's products are regulated could well set a precedent for the regulation of future pharmaceutical crops in California and elsewhere. If stringent standards are not established now, it may well prove more difficult to give future trials of pharm crops the degree of regulatory scrutiny they merit. Therefore, we urge the California Department of Food and Agriculture, the California Department of Health Services and the California Environmental Protection Agency to conduct their own independent review of the human health, environmental and economic concerns posed by Ventria's rice, including those raised in this letter. In the interests of prudence, we further encourage California authorities to establish a moratorium on the open-air cultivation of pharmaceutical crops, especially food crops.

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Human Lactoferrin: a Novel Therapeutic with Broad Spectrum Potential

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Abstract

Lactoferrin (Lf), a natural defense iron-binding protein, has been observed to possess antibacterial, antimycotic, antiviral, antineoplastic and anti-inflammatory activities. The protein is present in exocrine secretions that are commonly exposed to normal flora: milk, tears, nasal exudate, saliva, bronchial mucus, gastrointestinal fluids, cervico-vaginal mucus and seminal fluid. Additionally, Lf is a major constituent of the secondary specific granules of circulating polymorphonuclear neutrophils (PMNs). The apoprotein is released on degranulation of the PMNs in septic areas. A principal function of Lf is that of scavenging 'free' iron in fluids and inflamed areas so as to suppress free radical-mediated damage and decrease availability of the metal to invading microbial and neoplastic cells. Mechanisms of action of Lf in addition to iron deprivation also are described.

Administration of exogenous human or bovine Lf to hosts with various infected or inflamed sites has resulted in some prophylactic or therapeutic effects. However, an adverse response to the protein might occur if it were to stimulate antibody production or if it were to provide iron to the invading pathogen. The recombinant form of human Lf has become available. Development of the product for use in a considerable spectrum of medical conditions now can be anticipated.

Introduction

The iron withholding defense system, possessed by all vertebrate species, serves to scavenge and sequester toxic quantities of the metal. Consequences of over abundant body iron include catalysis of formation of excessive hydroxyl or ferryl radicals, suppression of various leukocytic defense mechanisms, and stimulation of growth of microbial and neoplastic cell invaders (Weinberg, 1993; Kontoghiorghes and Weinberg, 1995). A paramount component of the iron withholding defense system is lactoferrin (Lf). The recombinant form of the human protein has become available; development of the product for use in a variety of medical conditions now can be anticipated.

Lactoferrin, a 78-kDa glycoprotein, consists of a single chain of 692 amino acids folded into two globular lobes. Each lobe is conjugated to a 3-kDa glycan chain through an N-glycosidic linkage. The lobes each enclose a powerful iron-binding site; active residues are two tyrosines, a histidine and an aspartate. The apo form has an open conformation in which the iron binding site is near the protein surface. The iron complex is a closed system in which the metal exists below the protein surface and is inaccessible to the surrounding solution (Chug and Raymond, 1993).

Lactoferrin is structurally similar to transferrin (Tf) with about 44% homology. Like Tf, lactoferrin can bind two atoms of iron. For completion of the chelate rings, both Lf and Tf require bicarbonate ions. However, the affinity constant of 10^{24} for the iron complex is about 260 fold stronger than that of Tf. Moreover, unlike Tf, lactoferrin avidly retains the metal in acidic environments.

Functions of Lactoferrin

Iron binding in secretions

The two transferrins, Lf and Tf, function in a complementary manner to continuously purge body fluids of non-protein bound 'free' iron. Thus Tf is responsible for maintaining an enviroriment void of free iron in serum, lymph and cerebrospinal fluid. Lactoferrin is assigned to exocrine secretions that are commonly exposed to normal flora: tears, nasal exudate, saliva, bronchial mucus, gastrointestinal fluids, cervico-vaginal mucus and seminal fluid.

In addition to its iron removal function, Tf has an important second assignment conveyance *of* nutritional amounts of the metal to and from cells throughout the body. To accomplish the latter mission in humans, Tf normally maintains an iron saturation value of 25-35%. At values above 35%, Tf begins to lose its effectiveness as a scavenger of hazardous iron (Kochan, 1973; Weinberg, 1974). In serious episodes of infection, the iron saturation value of Tf can be reduced to as low as 5%. This action markedly enhances its ability to withhold the metal from invading pathogens.

Nutritional role (?)

In contrast to Tf, lactoferrin is not known to have a normal nutritional function (Sanchez et al., 1992). Its ability to retain the metal at mildly low pH values would prevent the protein from quickly releasing iron in acidic endosomes as occurs with Tf. In transferrin, interdomain hydrogen bonds are rapidly protonated to trigger opening of the iron cleft with prompt release of the metal (Abdallah and Chahine, 2000). In contrast, interdomain hydrogen bonds of Lf do not protonate in mild acidity and iron is retained at pH values >3.5.

Accordingly, in hosts, Lf fails to provide nutritional iron. For instance, in a study in 3-10 mos infants, Lf in breast milk was found to suppress rather than enhance iron absorption from the diet (Davidsson et al., 1994). Nevertheless, in unicellular systems, iron saturated Lf has been observed to stimulate growth of selected eukaryotes and prokaryotes. For example, Fe-Lf enhanced, and apo-Lf inhibited, proliferation of human enterocyles (Caco-2 cells)(Oguchi et al., 1995). Similarly, growth of *Legionella pneumophila* was stimulated by Fe-Lf and suppressed by apo-Lf (Byrd and Horwitz, 1991). The ability of cells of some bacterial species to bind Fe-Lf and to derive the metal from this sole source of iron is well established (Vogel et al., 1997).

Antimicrobial defense

Lactoferrin is a major constituent of the secondary specific granules of circulating polymorphonuclear neutrophils (PMNs). The apoprotein is released on degranulation of the

leukocytes in septic areas. In such sites, the pH value is lowered by catabolic acids released from metabolically active invading cells as well as from PMNs. With its ability to chalets and retain iron at low pH values, Lf is indeed a useful and probably an indispensable defense protein. For disposal of iron saturated Lf, hepatocytes might serve as a major depository (Brock et al., 1994).

Several investigators have noted the joint presence of Lf and lysozyme (LZ) in milk (Reiter, 1983), specific granules of PMNs (Ellison and Giehl, 1991), tears (Leitch and Willcox 1998) and tubotympanum mucus (Lim et al., 2000). In in vitro tests with *Escherfchia coli, Salmonella typhimurium and Vibrio cholerae*, each of the proteins alone was bactenostatic; together, they were bactericidal (Ellison and Giehl, 1991). In artificial tear fluid, synergy of Lf and LZ was observed against *Staphylococcus epidermidis* (Leitch and Willcox, 1998).

Some activities of Lf require prior conversion of the apoprotein to the ferreted molecule. In these systems, the mechanism of action would most probably be associated with the oxidant activity of the metal. Intro-macrophage killing of *Trypanosome cruzi* amastigotes and *Listeria monocytogenes* was enhanced by Fe-Lf (Lima and Kmerszenbaurn, 1987) as was suppression of infra-erythrocytic growth of *Plasmodium faldparum* (Fritsch et al., 1987). Human Fe-Lf arrested growth of breast carcinoma cells by inhibition of the G1 to S transition of the cell cycle (Damiens et al., 1999). Intra-peritoneal injection of either iron-saturated or apo-Lf suppressed growth of tumors in mice (Bezault et al., 1994). In systems in which serum is present, apo-Lf might obtain the requisite iron from transferrin (Fritsch et al., 1987).

Examples of other activities of Lf that are not concerned with iron deprivation include enhancement of (i) adherence of PMNs to endothelial cells (Oseas et al., 1981) and (ii) functions of natural killer cells (Shau et al., 1992); Bezault et al., 1994); Damiens et al., 1998). Lactoferrin also modulates the inflammatory process in part by preventing endotoxin activation of macrophage cytokine induction by binding to lipid A of lipopolysaccharide (Lee et al., 1998, Baveye et al., 1999). Administration of Lf prior to challenge with either endotoxin or bacterial pathogens can protect against septic shock (Lee et al., 1998; Baveye et al., 1999).

Lactoferrin has been reported to inhibit replication of such viruses as cytomegalo, hanta, hepatitis C, herpes simplex human immunodeficiency and poliomyelitis apparently by interfering with attachment of infectious particles to host cell receptors (Hanmsen et al., 1995; Marchetti et al., 1998; Tanaka et al., 1999; Voriand, 1999; Murphy et al., 2000). Inasmuch as metal binding causes a conformational change in Lf, the antiviral effect might be expected to vary with the percentage of iron saturation. In replication of human herpes virus in green monkey kidney cells, the $ID_{(50)}$ of bovine Lf saturated 10% with iron was 0.36µM; with 90% iron, 0.15µM (Manchetti et al., 1998). Iron, alone, had no antiviral effect

An antimicrobial function that does not involve iron trapping has been suggested for specific fragments of Lf. Pepsin digestion of human or bovine apo-Lf yields basic peptide sequences, distinct from the iron binding regions, that apparently after cytoplasmic membrane permeability of bacteria, fungi and protozoa. The peptides, termed lactoferricins, range in length from 10-47 amino acid residues.(Voriand, 1999). Release of the peptides in vivo might occur upon exposure of Lf to gastric pepsin or to pepsin-like proteases in neutrophilic phagolysosomes.

In in vitro tests, synthetic peptides corresponding to the first eleven residues of the N terminus *of* hLf have been observed to have strong bactericidal (Nibbering et al., 2001) and furgicidal (Lupetti et al., 2000; Ueta et al., 2001) activity. However, the size and tertiary structures of the synthetic peptides may differ considerably from those of natural peptides derived tam hLf (Nibbering et at., 2001). Possibly, potent antimicrobial peptides are formed from hLf at specific sites of invasion. Thus, in chemotherapy, hLf might be more effective than synthetic peptides (Nibbering et al., 2001). In a study in mice cited below, peroral hLf was more active than a synthetic peptide in reducing the level of urinary tract infections (Haversen et al., 2000).

Concentration of Lactoferrin in Body Fluids

Examples of the concentrations of apo-Lf in fluids of healthy and infected humans are contained in Table 1. The large amount of Lf in human milk suppresses growth of such iron-dependent bacteria in the infant intestine *as Bacteroides, Clostridium, Escherichia, Salmonella* and *Staphylococcus* (Weinberg, 2001). Accordingly, the gut of the breast-fed infant, in the absence of supplemental iron, develops a predominantly natural flora of non-pathogenic *Lactobacillus* and *Bifidobacterium.* The former totally abstains from use of iron; its enzymes utilize manganese and cobalt in place of iron (Weinberg, 1997). Growth of *Lactobacillus* results in a gut pH value of 5, whereas the gut pH of formula-fed infants is 5.9-8.2. Although *Bifidobacterium* requires iron, it has developed a unique ferrous iron acquisition system that can function at pH 5 and which, to a considerable extent, is resistant to iron withholding by apo-Lf. The fungistatic action of human milk has been shown to depend solely on its content of apo-Lf; the action is abolished by addition of iron (Andersson et al., 2000).

The large concentration of apo-Lf in tears, together with LZ, efficiently protects ocular tissues from most bacterial pathogens. These two natural (non-immune) proteins permit much lessen reliance on secretory antibody (IgA) for antibacterial defense. Accordingly, possible scanning of delicate ocular tissues due to antigen-antibody reactions is minimized.

Note also in Table 1 the remarkable increase in concentration of Lf in serum during severe bacterial infection. The protein is derived from degranulating neutrophils. One million neutrophils have been estimated to contain 3µg Lf (Bennett et al., 1973) Inherited inability to produce specific granules and neutrophlic Lf is associated with recurrent infection and, in untreated persons, death (Breton-Gorius et al., 1983).

Moreover, appearance of Lf in increasing amount in serum is a very early indication of an inflammatory reaction to invasion or trauma. For example, intravenous injection of E. colt in piglets caused a rise in serum Lf from 0.01 µM at zero time to 0.1 µM at 1 h and 0.2 µM at 2 h (Gutteberg et al., 1988). Similarly, in the initial phase of infection with *Neisseria meningitides,* serum Lf in humans increased at a rate of 0.15 µM/h (Gutteberg et al., 1984).

Table 1: Examples of concentrations of apo-Lf in human body fluids (μM)

Fluid	Concentration	Underlying condition	Reference
Colostrum	100	normal	Sanchez et al., 1992
Milk	20	normal	Hamosh, 1998
	40	normal	Fond et al., 1977
	60	normal	Zavaleta et al., 1995
Tears	25	normal	Hunt et al., 1996
Seminal fluid	1.4	normal	Buckett et al., 1997
Vaginal fluid	2.0	just after menses	Cohen et al., 1987
	0.1	just prior to menses	ibid
	<0.25	oral contraceptive users	ibid
Saliva	0.11	normal adults	Tenuvuo et al., 1986
	0.05	normal children	Smith et al., 1981
	0.25	children: cystic fibrosis	ibid
Amniotic fluid	0.02	non-infected	Pacora et al., 2000
	0.0	infected	ibid
Cerebrospinal Fluid	0.00	normal children	Maffei et. al., 1999
	0.01	Children: aseptic meningitis	ibid
	0.13	Children: bacterial meningitis	ibid
Synovial fluid	0.014	non-inflammatory	Bennett et al., 1973
	0.338	inflammatory arthritides	ibid
Serum	0.005	normal	Kelver et. al., 1996
	2.5	acute sepsis	Vorland, 1999

Administration of Exogenous Lactoferrin

The considerable spectrum of activities and locations of Lf suggest that the protein might be developed for a variety of prophylactic and therapeutic applications. A number of pilot studies are available that provide information on this possibility. For instance, intraperitoneal (i.p.) injection of rhLf in mice followed 10 h later by i.p. inoculation of *E. coli* decreased mortality from 43% to 0% (Wand et al., 1995). Administration of topical 1% bovine Lf prior to inoculation of herpes simplex type 1 on mouse cornea suppressed but did not eradicate the infection (Fujihara and Hyashi, 1995).

In Table 1, it may be seen that inflamed body joints might be another appropriate site for testing exogenous Lf. Examination of synovial fluid from 25 humans with inflammatory synovltis showed that 30% of the specimens contained 'free' iron. In these samples, concentrations of Lf were significantly lower than in those with no 'free' iron (Guillen et al., 1998). Addition of exogenous human apo-Lf to the samples consistently reduced the amount of 'free' iron. In a subsequent study, collagen arthritis was induced in DBA/1 mice and S. aureus septic arthritis was established in Swiss mice (Guillen et al., 2000). In each set, peri-articuiar injection of human Lt significantly reduced inflammation.

The systemic mechanism of action of orally administered Lf is not well understood. The extent to which available iron in the intestine might modulate the amount of Lf digested and/or absorbed is unclear. In 24 mice inoculated with *E. coli* by bladder installation and fed 0.5 mg hLf, serum samples at 24 h contained 0.02-1.1 nM Lf in eleven animals, none in thirteen (Haversen et al., 2000). Urine specimens, 2 h after feeding Lf, contained 0.5-1.0 nM; 5 h after feeding, 0.2-0.4 nM. In this investigation, bovine Lf and synthetic peptide sequence 16-40 also were
tested. perorally. The hLf-treated group showed the strongest reduction in numbers of kidney and bladder bacteria.

Prefeeding, on intravenous injection, of human and bovine Lf reduced kidney infections in mice inoculated with *S. aureus* by 40-60% and lowered viable counts 5-12 fold (Bhimani et al., 1999). In this study, apo- and holo-Lf were found to be equipotent, but hydrolyzed Lf was inactive. Daily feeding of bovine Lf to guinea pigs infected with dermatophytes failed to prevent onset of symptoms during the early phase of infection but facilitated clinical improvement of skin lesions after the peak of symptoms had occurred (Wakabayashi at al., 2000).

Development of various tumors in the colon, esophagus and lungs of rats exposed to chemical carcinogens was partially suppressed by feeding bovine Lf (Ushida et al., 1999). Orally administered bovine Lf inhibited angiogenesis in adult rats (Norrby et al., 2001). Prefeeding
bovine Lf to germ free piglets provided significant protection against lethal shock induced by intravenously administered endotoxin (16.7% vs 73.7% mortality, $p < 0.001$)(Lee et al., 1998).

Human patients with hepatitis C were fed bovine Lf for eight weeks (Tanaka et al., 1999). Three of four patients with low pretreatment levels of viremia experienced a decrease in serum values of HCV-RNA and alanine transaminase. However, no significant changes occurred in seven patients who had high pretreatment levels of viremia.

Exogenous Lf might be useful as an adjunct in antimicrobial therapy. In in vitro tests, effective concentrations of diverse antifungal drugs were lowered against *Pneumocystis cannii* (Cirioni et al., 2000) and *Candida* sp. (Kuiper et at., 1997; Wakabeyashi et al., 1998) by combination with either bovine on human Lf. Similarly, bacteriostatic and bactericidal concentrations of rffampin and doxycycline were lowered against *Pseudomonas aeruginosa* and *Burkholderia cepacia* by combination with rhLf (Alkewash et al., 1999). Of course, only pathogen: unable to employ Lf as an iron carrier could be safely attacked in this manner. To date, fungi are not known to extract iron from transferrins such as Lf. However, *Trichomonas* protozoa (Weinberg, 1999) and

Helicobacter pylori bacteria (Dhaenens et al., 1997) as well as various members of the bacterial family, Neisseriaceae (Vogel et al., 1997) can acquire iron from human Lf.

Production of human recombinant Lf has been reported in a variety of organisms. These include baby-hamster kidney cells (Stowell et al., 1991), *Aspergillus nidulans* and *A. awamori* (Wand et al., 1992, 1995), Saccharomyces cerevisiae (Liang and Richardson, 1993), transgenic dairy animals (Krimpenfort, 1993) and transgenic potatoes and tomatoes (Arakawa et al., 1999). In the *A. awamori* fermentation, quantities in excess of 25 μM have been obtained. The protein molecules are glycosylated and have excellent metal binding and antibacterial activity.

Possible Hazards of Exogenous Lactoferrin

A major advantage of human Lf over other chelating drugs is that it is a natural product of humans and thus should be biocompatible. **However, an important potential hazard of therapeutic use of hLf in human patients is possible induction of an antibody response.** Antibodies to endogenous Lf have been detected in patients with such autoimmune diseases as systemic lupus erythematosis, rheumatoid arthritis and primary scerosing cholangitis (Skogh and Peen, 1993; Alfetra et al., 1996). In some patients with rheumatoid arthritis, antibodies to Lf are present in synovial fluid as well (Guillen et al., 1998), Unfortunately, anti-Lf IgG can cause lactoferrin bound iron to become reactive in the bleomycin assay (Guillen at al., 1998).

A second possible hazard of exogenous human Lf is stimulation of growth of specific pathogens. As mentioned above, *Trichomonas vaginatis* obtains iron from human Fe-Lf. The protozoan grows mainly in the Lf-rich environment of human vaginal mucus. Disease symptoms begin or exacerbate during menses at which time the vaginal concentrations of Lf and iron are notably grater than at midcycle. In the male urethra, the illness is self-limited or asymptomatic;
seminal fluid contains Lf but is vary low in iron, as is urine. Fortunately, *T. vaginalis* cannot obtain iron from Tf and thus fails to cause systemic infections in either women or men (Weinberg, 1999 Were human Lf to be used in therapy of vaginal yeast infections, the patients would first need to be carefully evaluated for freedom from trichomoniasis.

Another human pathogen that can specifically derive iron from human Lf is *Helicobacter pylori*. This bacterium is the major etiologic agent of chronic gastritis and is a component of the eptiology of gastric ulcers and carcinomas. Cells of this pathogen form a 70 kDa hLf-binding protein. *H. pylori* also can obtain iron from heme but mot from human Tf nor from bovine or equine Lf or Tf (Dhaenens et al., 1997). Thus bovine Lf can suppress *H.* pylori infection in mice (Dial et al., 1998; Wada et al., 1999).

The singular location of *H. pylori* in human gastric epithelium apparently is a consequence of the availability of human Lf and iron in gastric juice. In a set d 30 *H. pylori* positive and 14 negative patients with chronic gastritis (Nakao et al., 1997), the average level of endogenous Lf in the former was 4.25 fold greater than in the latter ($p<0.0007$).

In in vitro susceptibility tests of *H. pylori* to apo-rhLf, 5 of 13 strains required 10 μM for inhibition, 3 required 20 μM, and 5 needed >40 μM (Miehlke et al., 1996). No strains were sensitive to low concentrations of the protein. **Thus human apo-Lf might not be a successful therapeutic agent for *H. pylori* and, indeed, could intensify the infection.**

[apo-Lf is lactoferrin without bound iron: BF] In a recent study, six adult humans with *H. pylori* infection were fed 1.25 g and six were fed 5 g of rhLf over a 24 h period. The amount of endogenous Lf had not been ascertained. Not surprisingly, none of the 12 subjects cleared the infection. Fortunately, no adverse effects were observed (Opekun et al., 1999).

In contrast to *H. pylori*, *Helrcobacter felis* does appear to be susceptible to the antibacterial action of human Lf. In mice infected with $3x10^9$ viable cells of the latter bacterium, rhLf partially reversed both the infection-induced gastritis and the infection rate (Dial et al., 2000).

In this system, the efficacy of Lf was comparable to that of amoxacillin as well as to the combination of metronidazole, tetracycline and bismuth subsalicylate.

Perspectives

As rhLf increasingly becomes available, it may be appropriate for best results to substitute this product for bovine Lf in studies in humans. However, in some systems, bovine Lf has been observed to be more effective than human Lt. For instance, the ability of *Prevotella nigrescens*, a bacterium associated with dental infections, to adhere to am enamel component, hydroxapatke, was suppressed to a greater extent by bovine than by human Lf (Yasuyuki et al., 2001). In a study of the cytopathic sited of human immunodeficiency virus-1 on MT4 cells, the IC(50) of bovine Lf was 0.5 µM; of human Lf, 1.0 µM (Harmsen et al., 1995).

Furthermore, as with any iron chelator, it will be essential to recognize and monitor the iron background of the system under investigation. Effective doses of Lt would be expected to vary with the level of iron available to the protein. The degree to which, if any, iron might be required by Lf for the specific activity also should be determined. With the exception of the investigations on synovial fluid (Guillen et al., 1998), the pilot studies cited above failed to ascertain tissue or fluid values for endogenous Lf and iron.

In systems in which Lf were to be employed as an adjunct to, or a replacement for, antibacterial drugs, consideration should be given to pairing it with lysozyme. However, disease states that probably would not be helped by lysozyme include fungal and viral infections, cancers, and sterile inflamed areas.

Ten years ago, Sanchez et al. (1992) proposed that the 'biologic role of Lf is that of a specialized iron-scavenging protein, designed to act particularly under conditions where Tf would be less effective at binding iron due to reduced pH, such as exist in the gastrointestinal tract or inflammatory lesions. By binding iron under these conditions it would render harmless 'free' iron that might otherwise cause free radical-mediated damage to sensitive tissues, reduce absorption of iron in the immediate post-natal period, and decrease its availability to microorganisms'. During the past decade, successful research applications of administration of exogenous Lf have tended to validate this proposal. In the coming decade, some of these applications might well be developed into prophylactic or therapeutic products.

Conclusions

Lactoferrin, a 78 kDa iron binding protein, provides antioxidant and antimicrobial activity in secretions of lacrimal and mammary glands and of respiratory, gastrointestinal, and genital tracts. It is released also from neutrophils at sites of infection and can scavenge non-protein bound iron in areas that have lowered as well as neutral pH values. Recombinant human Lf is becoming available for evaluation for possible prophylactic or therapeutic use in a wide variety of human medical conditions. As a human natural product, it should be efficiently metabolized with no side effects. Precaution is needed, however, to avoid antigenic sensitization as well as introduction of the protein to tissues that may be infected with specific protozoa or bacteria that utilize Lf in their acquisition of host iron.

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Appendices in the letter from Frank E. Young to Lester M. Crawford dated July 29, 2005.

The letter from Frank E. Young to Lester M. Crawford dated July 29, 2005 is referred to as Young to Crawford

The previous letter from Peter Barton Hutt of Covington and Burling to Laura M. Tarantino, Robert L. Martin, and Barbara O. Schneeman, dated July 19, 2005, is referred to as Hutt to Tarantino, et al.

Appendix 1 of Young to Crawford corresponds to Attachment 4 of Hutt to Tarantino et al.

Appendix 2 of Young to Crawford corresponds to Attachment 5 of Hutt to Tarantino et al.

Appendix 3 of Young to Crawford corresponds to Attachment 3 of Hutt to Tarantino et al.

Appendix 4 of Young to Crawford corresponds to Attachment 6 of Hutt to Tarantino et al.