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OF PREVENTIVE VACCINES:

RECENT ADVANCES AND REGULATORY CONSIDERATIONS

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C O N T E N T S

PAGE

MORNING SESSION:

INTRODUCTORY REMARKS:

MARGARET ANN MILLER

Office of Women's Health, FDA

4

REPRODUCTIVE TOXICITY ASSESSMENT
OF PREVENTIVE VACCINES

PRESENTATIONS:

[Note: All presentations were
accompanied by slides]

REPRODUCTIVE TOXICITY ASSESSMENT
OF PREVENTIVE VACCINES:

MARION GRUBER, Ph.D.

Office of Vaccines Research & Review, FDA

16

HUMAN T AND B CELL DEVELOPMENT:

RICHARD INSEL, M.D.

Univ. of Rochester, School of Medicine

54

MATERNAL IMMUNE SYSTEM STIMULATION
& EFFECTS ON FETAL TERATOGENESIS:

STEPHEN HOLLADAY, Ph.D.

College of Veterinary Medicine,
Virginia Polytechnic Institute

94

DEVELOPMENTAL IMMUNOTOXICOLOGY:

RALPH SMIALOWICZ, Ph.D.

U.S. Environmental Protection Agency

131

AFTERNOON SESSION:

ROUNDTABLE DISCUSSION:

MERCEDES SERABIAN, CBER, FDA, Moderator

MARION GRUBER, OVR, FDA, Moderator

161

TOPIC ONE: "Study Design"

MILDRED S. CHRISTIAN, Ph.D.

Executive Director of Research

Argus Research Laboratories

165

C O N T E N T S [Cont'd.]

	<u>PAGE</u>
TOPIC FOUR: "Animal Models" PAUL BARROW, Director, Toxicology, MDS Pharma Services Preclinical	186
QUESTION & ANSWER SESSION	199
TOPIC TWO: "Immunological Endpoints" FRANCOIS VERDIER, Pharm.D., Ph.D. Product Safety Assessment, Aventis Pasteur	258
TOPIC THREE: "Developmental Endpoints" JAN-WILLEM VAN DER LAAN, Ph.D. Director, Pre-Clinical Assessment Group, Medicines Evaluation Board [RIVM], The Netherlands	262
SUMMARY QUESTION & ANSWER SESSION	264

M O R N I N G S E S S I O N

1A

MS. MILLER: I'm your lead-off speaker this morning. I am Margaret Miller. I am with FDA's Office of Women's Health, and our office is located in the Office of the Commissioner. And our mission is to serve as a champion for women's health, both inside and outside the agency.

And it is indeed a pleasure to help sponsor this meeting. I was very pleased with the discussion yesterday, and I think we'll have more fruitful discussion today.

I would like to say that Christine Everett of our office was involved in organizing this meeting. So if you have any complaints, I'd ask you to direct them directly to her.
[Laughter.]

MS. MILLER: One of the main reasons why our office came into being was to encourage the participation of women in clinical trials for products that would be used by both men and women. And the current guideline on the participation of women in clinical trials was written in 1993. And it does recommend that women participate in all phases of clinical trials, and this includes women of child-bearing potential; that we look at the data by sex and we analyze for gender differences, to see if the product acts differently in men versus women.

Now, while we recommend the participation of women in clinical trials, we do still have concerns about fetal

safety. And so the recommendation does not extend to pregnant women. So regarding the participation of pregnant women in clinical trials, this is really limited to those products which are intended to treat a condition that occurs only in pregnancy.

Well, this leaves us with a problem. Because while we are not including women in clinical trials except for those cases where it's used to treat pregnancy, we know that women get sick. Women get influenza while they're pregnant. And treating a pregnant woman often confers benefit not only to her, which is our office's concern, but to the developing fetus as well. But yet, at the time of an approval we don't have information on fetal safety, or even on what health benefits or differences that product might have in a pregnant woman versus a non-pregnant woman. To add to the problem, we have the fact in this country that about half of all pregnancies are unintended; which means many women are having therapies and different treatments without knowing they're pregnant. And at the time that they realize they're pregnant, they go back through their mind and go through all those things they've done for the past month or so. And they come in and they say, "How will these activities affect my baby?" And that's a big question for them.

So when a clinician is trying to treat a pregnant woman or a woman of childbearing potential, they really want to

balance the health benefits of a product versus the safety concerns for both the fetus and the mother. And in order to do this, the agency has recognized that this is an area where we really need to do a better job in providing clinicians and women with this type of information.

One of the activities that the agency is undergoing is an effort to revise the labeling section of our products. And this has been an ongoing concern that the health care community has brought to the agency: that the label--the way it's formatted and the information that is imparted-- does not provide them with the type of information they need to make clinical decisions. So that is an ongoing effort.

But as we started in this effort, it became very clear that reformatting bad information is just bad information reformatted. And really, there needed to be a concerted effort to improve the content, or improve the information that we were putting into the pregnancy labeling.

So the past three years, our office, together with our colleagues in the centers, have been working at ways of improving the content; giving those fetal safety concerns that I've already talked about, and understanding the limitations of doing studies in pregnant women. We've tried to look at novel and creative ways of getting good information for pregnancy.

The first activity is, the office has actually funded some PK studies, doing studies in pregnant women. I'll talk a little about that.

We have created a pregnancy registry website, to encourage women to participate in ongoing pregnancy registries.

And then, the third activity, which is why we're here today, is that we're interested in: How can we do a better job of using animal models to make predictions so that we can give women good information?

Let me talk a little bit about the ethics of doing studies in pregnant women. As soon as we start talking about enrolling pregnant women in clinical trials, everybody gets this glazed look of panic, "deer in the headlight" type of approach. And I will agree that it is not as easy to do studies in pregnant women as young, healthy, male volunteers. That is a fact.

We do have ethical rules regarding the conduct of clinical trials. And they specifically address pregnant women. The basic human subject protections for federally-funded research are found in 45 CFR Part 46. Subpart A is your basic protections for all subjects. Subpart B covers the pregnant women, the fetus, and in vitro fertilization.

Let me just walk you through some of the highlights of this regulation. The first, Subpart A does allow for expedited review for something that is minimal risk. So if you have a study and you say, "Well, it really involves minimal risk

to the participants," under Subpart A you can get expedited review.

Unfortunately, if you are doing a study in vulnerable subjects--and that's children, pregnant women, prisoners, mentally disabled, or economically disadvantaged people--you cannot get expedited review. So you're in for full IRB approval when you're doing a study in pregnant women.

In addition to all the criteria under Subpart A, when you are doing a federally-funded study you have to comply with Subpart B. And Subpart B was changed about a year ago.

I'm going to talk about the new regulations.

And that says that pregnant women can give informed consent and can participate in the trial, if the following conditions are met. Now, the first is that we have done studies in non-pregnant women. And the second is that we've conducted animal studies.

Now, the regulation does not dictate that those animal studies be developmental studies. But I have taken four proposals through IRBs, and I can assure you that's what the IRB asks us. They are asking for developmental animal studies, in order to write an informed consent document that the woman can make a decision about whether or not she wants to participate in this trial.

Finally, or next, if the research is designed to meet the health needs of the mother, and the risk to the fetus is

minimal or the minimum that we can obtain with the study, then the maternal consent alone is necessary.

Also, for studies where we're going to benefit the mother and the fetus, or we're just adding to general knowledge, then material consent alone is all that's required for the woman to participate in the trial.

However, if you are designing a federally-funded research study and the aim of that study is to provide a treatment which is designed to benefit the fetus only--some type of vaccine, or you're just using the mother as a vehicle and the benefit is going to be only to the child--then you need to get consent from both the mother and the father in order for the woman to participate in the trial.

Now, I would like to mention, one of the questions we get sometimes is, we know that once we approve a product that it is going to be used by pregnant women. Why don't we just collect the information from that study, and make decisions about fetal safety that way?

And certainly we have asked for pregnancy registries on a number of products. These are phase IV studies where, once a product is approved and it finds its way into pregnant women, we ask those women to enroll in a registry. And generally, we'd like to see them enroll after they've been exposed, but before the birth outcome is known. And this is a very good tool for collecting safety information, both

on the mother and on the infant. Because we can examine those with time.

Well, what we've found out is that, while the agency has been asking for these studies for a number of years, this was the best-kept secret of women's health: that we would go to the advocacy communities; they were not aware of pregnancy registries. If you talked to women about pregnancy registries, they just did not know about this activity.

So one of the things our office has done recently is we've put together a pregnancy registry website. And this is a website that encourages women to take needed medications, not to be scared off medications that they need to maintain their health; and then to participate in a registry.

And so we have a list of all ongoing registries that are for medicines that women need to maintain their health.

And it is to encourage their participation. And we also have a "Contact Us." So if you have a registry that you would like to have included on our list, you can send us an e-mail and we'll incorporate that into our registry list.

So while we have tried all these tools, we come down to the fact that animal models are still going to be the main type of information that we will have for most products when they are approved. We are not likely to see women enrolling in clinical trials any time soon. And even if we wanted to, in order to give a woman informed consent, you

need to have some of that animal model to base your prediction on.

And I think--and we heard some of this yesterday--that even if we had registries for everybody, we're limited as to the type of information that you can get from a registry.

You're not going to necropsy those babies and do lymph nodes. You know, it's just not going to happen.

So if you don't know what you're looking for in a registry, you're going to maybe look for major malformations.

There's problems with long-term follow-up. Really, you need the animal models to give you the signals, even to design a good registry: What should we be looking for?

So that brings me to the challenge for this group, and I know you're up for it. Because we do not want to design animal studies to make predictions for animals. Really, what the women need and what women want to know is: How do we interpret that finding in animals to the human situation?

And certainly that is the challenge for this group. And after the discussion yesterday, I'm sure you're up for it. So I'll turn it over to Marion.

[Applause.]

DR. GRUBER: Well, I really would like to thank Peggy Miller for these very nice, very right-on-target introductory remarks.

And I just wanted to ask, if you throw this against the wall, does he turn into a prince? [Referring to slide of frog.]

DR. GRUBER: Okay. Well, before I start my presentation, I think I have to do some--or I was asked to inform you about the most important issue first. And that is that lunch today is in the Montecello Ballroom, again on the dining level upstairs. And I think you can also take the stairs, and don't have to wait for the elevators to go up there. The other thing I need to remind people of is to use the microphones when they have questions, and to introduce themselves.

We will make available the presentations, the slide presentations of the speakers, following this meeting of the SOT, once they have received all the slides from the speakers. We'll make them available about two weeks after this meeting. And I think we're also thinking about having an evaluation form that you can then fill out by e-mail. So having said that, and I hope I didn't--No, I forgot a lot of things. I was told to thank all the speakers and panel members yesterday for a very fruitful and helpful discussion. That has been tremendously helpful for FDA, and we think we have an idea really how to actually begin to think about guidance. Let's be careful.

And I also wanted to thank again, as Karen Midthun did yesterday, the SOT, and especially Shawn Lamb and her

staff, for making this a very smooth, easy-going workshop. So thank you very much, to Shawn and her staff, for helping us with this.

So I think I'm ready then for my presentation.

REPRODUCTIVE TOXICITY ASSESSMENT

OF PREVENTIVE VACCINES

PRESENTER: MARION GRUBER, PH.D., OVR, FDA

DR. GRUBER: So for those who don't know me yet, my name is Marion Gruber. I'm with the Office of Vaccines. And I have been actually involved over the last couple of years to try to generate policy and guidance for preclinical safety evaluation of preventive vaccines. And today's discussion will focus on reproductive toxicity assessments of preventive vaccines.

As you know, the FDA had announced in the Federal Register on September 8th the availability of a draft guidance document for industry that is entitled "Considerations for Reproductive Toxicity Studies for Preventive Vaccines for Infectious Disease Indications."

The guidance was published with the intent to provide sponsors with information regarding assessments of the reproductive toxicity potential of preventive vaccines that are indicated for maternal immunization, and to target populations that would include females of reproductive age. This document was generated and written when there was relatively little experience with performing reproductive

toxicity assessments for these types of products. And there was virtually no scientific literature to really assess and address this issue.

So since publication of this guidance and since the initiation of reproductive toxicity assessments in a more systematic way for some of these preventive vaccines, there have been a number of concerns and questions raised by sponsors, by experts that need to conduct these studies, and also by CBER reviewers which are then in the position to evaluate the data.

And many of these questions and concerns are also reflected in comments that we have received from industry in response to publication of this guidance. And the suggestion was made that a discussion should take place by experts in the field addressing reproductive toxicity studies for preventive vaccines, to further pioneer this relatively novel area.

So the goal and purpose of this second day of the workshop is then to discuss the technical aspects, the experimental designs, and the animal models for developmental or, let's say, reproductive toxicity assessments--I will get to the difference between those two in a little while--in order to reach a consensus on how to best perform these types of studies for preventive vaccines, and the type of information that can be derived from these studies, to

assure that it will be relevant and useful to better assess, and perhaps predict, human risk.

So today's discussion will serve to define the scientific challenges that one is faced with when having to conduct the studies. And I hope that we will define approaches as to how to overcome these challenges.

So I think the goal needs to be to try to define the most practicable and feasible designs that can be conducted in a reasonable manner. And because of the complexity of the issues that we are facing when looking at reproductive toxicity assessments for preventive vaccines, I don't think that we are able to get answers and reach consensus on all the aspects and questions that have been raised. But CBER is intending to revise the guidance document, after considering the comments, recommendations, and suggestions that we're going to be hearing from you today.

So the purpose of my presentation then will be to provide an overview of the past and current situations regarding immunization during pregnancy; to discuss the regulatory considerations and concerns regarding reproductive tox assessments for vaccines, and why we think that these studies are necessary; to provide an overview of the current version of the guidance document, so that we're all going to be on the same page; and to summarize the comments that we have been receiving from industry in response to publication of this guidance.

I will finish this presentation with questions that could form the basis for our discussions this afternoon.

Vaccination of pregnant women to protect mother and infant from infectious disease has been practiced worldwide for decades. And the most famous example perhaps is maternal immunization with tetanus toxoid vaccine, that has been very successful in preventing neonatal tetanus in developing countries.

Polio vaccine was given routinely to pregnant women in the United States in the late 1950s and the early '60s. And other vaccines were administered to pregnant women, especially in outbreak situations. And the one worth mentioning I think is the small pox vaccine; which is why today we have a lot of clinical experience and clinical data in assessing the clinical experience when you use small pox vaccine in immunizing pregnant women. And of course, these data I think are still going to be paramount in deciding the safety of even the new candidate vaccines that we have today.

Now, most vaccines that are currently licensed in the United States are not indicated for use during pregnancy. But depending on the vaccine, vaccination programs do frequently include pregnant women. For example, as Peggy addressed, the inactivated flu vaccines are often recommended for use in pregnant women in their second and

third trimester of pregnancy. Those women were at special risk for serious consequences from the flu.

In addition, there are also a number of vaccines that are recommended for use in pregnant women. This would include hepatitis A and B vaccines and meningococcal vaccines in situations of epidemic and endemic exposure. And these are recommended by the Advisory Committee for Immunization Practices.

The general approach of the Advisory Committee for Immunization Practices has been that the benefit of vaccination of pregnant women usually outweighs the risk when the risk for disease exposure is high, when infection poses a special risk to mother and fetus, and the vaccine is unlikely to cause harm.

Now, maternal immunization provides a strategy to protect young infants from severe infectious diseases through the passive antibody transfer from mother to fetus. And maternal immunization trials have been and are currently conducted in the United States to assess the safety and tolerability of vaccines against pathogens such as respiratory syncytial virus, streptococcus pneumonia, and group B strep.

And there are a number of controlled clinical trials that have been conducted. And they provide evidence that maternal immunization with at least inactivated vaccine antigens, including haemophilus influenza type B and

pneumococcal polysaccharide vaccines, appear to be well tolerated in the pregnant women and in their offspring. But I think what needs to be stressed is that these studies were usually not powered or designed to detect rare adverse events, or to assess long-term follow-up of the offspring. Even though there may not be hard evidence of reproductive toxic effects in humans caused by the use of currently approved vaccines, when assessing the preclinical and clinical safety of a candidate vaccine regulatory considerations take into account not only past experience, but also theoretical concerns.

So the regulatory approach does not presume a product to be safe until directly tested. And that is because the potential for an unexpected clinical adverse event can never be ruled out.

And addressing these concerns using the best available methods that are available to us is critical; in particular, as mentioned yesterday, in a public climate where the expectation is no risk, as the vaccination benefit may not be immediately obvious because of the relative absence of disease in our society.

The current situation is, as Peggy pointed out, unless the vaccine is specifically indicated for maternal immunization--that is, indicated for immunization of pregnant women--no data are collected regarding the

vaccine's safety in pregnant women during the pre-licensure phase of a vaccine.

But as more women participate in clinical trials, and as more preventive vaccines are being developed for adolescents and adults--and as an example, I'd like to mention human papilloma virus vaccine or HIV vaccine--there is increased concern for the unintentional exposure of an embryo or fetus before information is available regarding the potential risk versus benefit of the vaccine in general.

Also, use of licensed vaccines in females of childbearing potential would likely result in the inadvertent exposure of pregnant women and their fetuses to the vaccine, especially if you consider that about half of the pregnancies in this country are unintended. So it would be unlikely that a vaccine exposure would be avoided in these pregnancies prior to their clinical recognition.

Also, there is the situation that following approval vaccines which do not have specific regulatory approval may be recommended for use during pregnancy by public health policy makers.

Now, the potential risks that are involved in prenatal immunization programs overlap with those that we have been discussing yesterday. And I would include adverse events caused by the constituents of the vaccine; that is, potential intrinsic toxic properties of the vaccine

antigens, stabilizers, adjuvants, preservatives, and also potential adverse events that are caused by the immune response.

So it is conceivable that an immune modulation in the mother caused by vaccination during pregnancy could influence embryo/fetal development. And recent studies in animal models provide evidence for a maternally-mediated mechanism influencing fetal development. And we're going to be hearing some of these data today.

In addition, maternal immune modulation could influence the development of the immune system of the immature organism. And lastly, maternal immune modulation has been shown to influence the course and outcome of pregnancy.

In contrast to perhaps what we've have, or what the situation was in the last couple of decades, in our days we have a broad range of vaccines that are currently in clinical trials. And they have been discussed yesterday, and they are listed on this slide.

And these products are formulated with novel adjuvants, excipients, stabilizers, and preservatives. And they are frequently administered by new routes of administration. And for some of these products there is little preclinical and clinical experience.

And many of these products are indicated for adolescents and adults, which of course includes females of reproductive age. And some of them are specifically

indicated for the prevention of sexually-transmitted diseases. And we think that underscores the need for a more systematic approach to preclinical toxicity assessments, including reproductive toxicity assessments. Now, until recently, few or no licensed vaccines have been tested for reproductive and developmental toxicity in animals prior to their use in humans. But there is concern that there are no data to address developmental risks in pregnant women or women of reproductive age at the time of licensure of a preventive vaccine product.

And reproductive toxicity studies in animal models may offer one approach to identify potential developmental hazards. And we think that they are justified, as the target population for vaccines often includes women in their reproductive years who may become pregnant during the time frame of vaccinations; because clinicians are not infrequently confronted with situations where immunization of pregnant women may be beneficial. And lastly, vaccine labeling must have a statement about use during pregnancy. And as Peggy discussed, the FDA has a current initiative ongoing where it is proposing to amend its regulations concerning the format and content of the pregnancy subsection of the labeling for human prescription drugs and biological products.

And this rule would not only eliminate the current pregnancy categories, but the rule would require labeling

to include a summary assessment of the risk of using a product during pregnancy and lactation. And it would require a broader discussion of the data--and that is animal and human--that would underlie the evaluation of risks associated with a product.

And for all of these reasons that I've discussed, we have developed a policy for reproductive tox studies for vaccines that are indicated for maternal immunization and immunization of women of childbearing potential. And we have published this draft guidance document in September of 2000.

And I would like to now turn to providing you an overview of the guideline, as this is going to be the subject of our discussion this afternoon. And I also wanted to give you an idea about the comments that we have received from you in response to publication of the guideline.

And the way I thought I'm going to do it is I'm going to divide the guidance into different sections. And I will tell you about what the guidance states, and then at the same time, what comments we received from industry. So that we're going to be all on the same page in discussing the issues this afternoon.

You should note that industry comments represent several different points of view. And there are going to be apparent contradictions. But I decided to present those to you, to give you a true representation of the various

issues and concerns and questions that have been raised. And I think this will certainly spark a lot of discussion, but I would like actually for you to hold your comments and questions until this afternoon, because this is when we are looking at the different issues.

Now, starting with the guidance and the section on general considerations, the guidance states that each vaccine needs to be evaluated on a case-by-case basis, where product features and intended clinical use need to be taken into account when you design developmental tox studies.

If you have clinical experience that is derived from immunization of pregnant women, then this experience or the data and the outcome may be considered for any potential application in the design of the reproductive tox study.

All data that you may have from acute or repeat-dose preclinical tox studies should be reviewed for their possible contribution to the interpretation of any adverse developmental effect that you may observe in the developmental tox study. An example provided was fetal toxicity secondary to maternal toxicity.

The guidance also states that sponsors should use as a point of reference in the design of reproductive tox studies the ICHS5A guidance document published in '94, that is entitled "Guideline on Detection of Toxicity to Reproduction for Medicinal Products."

And since some special concerns are effects of vaccine exposure on the developing fetus, CBER had recommended studies to evaluate the effects on embryo/fetal development, so that the vaccine is administered during the period of organogenesis. That means that the female should be exposed to the vaccine from implantation to birth. And these studies are defined as stages "C" and "D" in the ICHS5A document.

But as we know with vaccines, many times modifications to dosing schedules are necessary to allow an antibody response to occur in an animal model. And so we also included in the guidance that priming doses may need to be administered prior to conception.

And we had also recommended to extend the stages "C" and "D" evaluations to also look at the period between birth and weaning, defined as stage "E" in the ICHS5A document, so that mother and offspring can be followed postnatally. So what did industry have to say? Actually, the majority of comments supported that, in principle, developmental tox studies for preventive vaccines are needed for vaccines that are indicated for maternal immunization and females of reproductive age; and that thus, efforts should be made to assess the risks of vaccination during pregnancy in animal models.

At the same time, however, we did receive comments questioning the relevance of developmental and reproductive

tox assessments in animal models for preventive vaccines. And among the major hurdles cited were the species specificity of the immune response combined with species specificity of developmental time lines in animals versus humans. And this would make the characterization of a relevant animal model very difficult and would, de facto, question the value of developmental tox studies for preventive vaccines.

Industry also thought that the guidance should clearly indicate that developmental studies to assess the potential adverse events on the female and developing conceptus from implantation through birth and weaning are sufficient, and that fertility studies and post-weaning assessments are not required.

Furthermore, the comment was made that it was not clear whether some of the endpoints are consistent with ICH reproductive toxicity guidelines. And we were asked to really rename the developmental endpoints stated in our guidance for consistency with the ICH document.

Then it was felt that the title of the document was somewhat broader than its scope, as classical reproductive toxicity assessments do include studies to assess impact on fertility and post-weaning assessments. And the suggestion was made for the document to refer to embryo/fetal toxicity, rather than to reproductive toxicity. But

additional guidance on the aspects of female fertility studies was requested.

Turning back to the guidance document, the section on immunological parameters and follow-up, the guidance states that the reproductive tox studies should be designed to include the detection of antibody production in the pregnant animal, and to also look at the feasibility of antibody transfer from the pregnant female to the fetus through antibody measurements in the fetus and newborn. We also thought that the antibody response in the fetus should be studied, looking at presence, persistence, and effects; including potential cross-reactivities with the antibodies induced in the pregnant mothers with fetal tissues.

And the guidance further stated that these studies should include an in-life phase, as I mentioned; a follow-up of the pups from birth to weaning, to evaluate further on the maternal antibody transfer to offspring; the magnitude and even persistence of antibodies in newborn pups; if you have presence of antibody in milk; and the effects of antibodies in the newborn. Potential interaction with host tissue was named again. We also listed some other endpoints, such as neonate adaptation to extra-uterine life, and the study of maternal behavior.

Industry says that in general there is agreement that it is important to demonstrate an immune response to the vaccine

in the dam, to demonstrate exposure. And the ability to detect an antibody transfer from the dam to the newborn was viewed as a key issue by some. And the suggestion was made that the proper species for a developmental tox study be validated in a preliminary study with only immunological endpoints.

But in general, it was felt that an extensive characterization of antibody responses in the dam and the fetus and neonates was not warranted, especially if no developmental toxicity is observed. So it should not be necessary to evaluate the immune response in greater detail.

The rationale for kinetics assessments was questioned, especially when the vaccine is not intended for pregnant individuals. Kinetics assessments in particular were viewed as challenging, as one litter would be required per time to obtain enough serum to really measure the immune response. And this would impact sample size. And also, there would be a lack of validated assay for measuring immune functions in newborns. If we would indeed request kinetics studies, we should really address how long-term kinetics should be followed.

One comment questioned the "appropriate immune response" in an animal model, as antibody generation would be only one factor. Cytokines, cell-mediated immune responses could

also result potentially in toxic effects; each with their own specific time lines.

Furthermore, the evaluation of potential cross-reactivity of maternal antibody with fetal tissue was viewed as an excessive burden, and not justified as long as no malformations or other effects would be observed.

The argument was made that if an antibody would have an adverse effect on fetal development, then it would likely be detected as effects on viability, growth, function, or other fetal abnormalities.

It was, however, suggested to include perhaps a broader histopathology assessment in developmental toxicity studies for preventive vaccines, as a measure to assess potential effects of maternal antibody on fetus or newborn animal.

And the suggestion was also made to conduct antibody assessments, including potential cross-reactivity assessments of maternal antibody with fetal tissue, as a tiered evaluation; that is, if you observed developmental toxicity, then you would conduct further studies to look at the mechanism of the effect.

Guidance was sought by industry on how long the offspring should be followed post-parturition. And we were asked to specify the end of the postnatal period for the most frequently used species.

Furthermore, a comment was made that body weight is the best indicator for pre-weaning developments, and functional

studies are not commonly conducted in pre-weaning pups, due to the limited repertoire of responses and difficulty in the quantitation of those responses.

Let's discuss another very easy issue, and that is the dose. Reproductive tox studies should include a dose response component, states the guidance, to be able to assess potential toxic effects that a particular dose may have on the dam or the fetus, to define a safe dose, and to look at the dose that is able to mount or to induce an immune response.

The guidance states the dosing regimen should include a full human dose equivalent, and that a dose scaled down because of feasibility considerations should ordinarily still exceed the intended human dose by at least fifteen-fold on a milligram-per-kilogram base.

The following comments were provided by industry on the issue of dosing. Dose range is not warranted, but the administered dose should induce an immune response in the species selected, and the dose should exceed the human dose on a milligram-per-kilogram base.

It was felt that the principles outlined in the documents for dose selection would refer to the notion of a classical dose response; whereas many immune-based reactions would not follow such a relationship. And also, the pharmacodynamics of immune reactions would be difficult to scale between an animal species and a human.

So with vaccines there may be also limits to the amount that can be administered, and frequently dose levels are often based on the volume of the material.

Then we were asked to clarify why we asked for an at least fifteen-fold greater than the human dose on a milligram-per-kilogram base. And there was one suggestion that doses may be defined in separate experiments in non-pregnant animals. But there seemed to be general agreement to use a single high human dose equivalent, if possible, for these studies.

What about immunization scheduling and exposure? The immunization interval and frequency of immunization, states the guidance, should be based on the clinically proposed immunization interval whereby a compressed scheduled would need to be allowed.

So episodic dosing would be more relevant than daily dosing, because it would mimic the clinically administered immunization schedule. The guidance states that modifications to dosing frequency may be necessary, depending on the kinetics of the antibody that is induced in the species, and also, when considering the length of gestation of the particular animal model.

We had only one comment from industry regarding immunization schedule and exposure, and that was loaded. The relationship of dosing to developmental timing will be

one of the most difficult aspects in designing developmental tox studies.

The point was made that there are potentially different responses in the host to initial priming doses, versus subsequent doses, versus eventually booster dosing. And the differences in responses could be reflected in different immune responses, such as antibody production, cytokine production, cell-mediated immunity. This would be compounded by species-specific developmental time lines. And having to tease these various issues out would make a study become unreasonably large and complex.

Animal models. The guidance document states that every effort should be made to select the relevant animal model. And we define it as the vaccine should be able to elicit an immune response in the animals.

The guidance states that the reproductive tox studies should not necessarily, or does not necessarily need to be conducted in the traditional species that are commonly used for reproductive tox studies--that is, rats and rabbits. And there is also no need for a specific requirement for the routine use of two species, like one rodent and one non-rodent. But it would be important to provide a rationale for the choice of the animal model that is proposed.

The guidance document further states that if there is no relevant animal model, then reproductive studies should be

done regardless, to assess the intrinsic potential of vaccine antigen. And I think we need to really discuss this this afternoon: what to do if we don't have a relevant animal model available to us.

Industry concurred that only one species should be required for developmental toxicity studies, and that the species should be able to mount an immune response to the vaccine. However, comments were made that we have only a limited number of animal models available, especially if we would include postnatal assessments, and especially when you consider species that have reliable background data and for which we have a lot of historical experience. And the question was raised of how to validate non-traditional species, and how much historical background data would be needed.

In terms of vaccine product class, or vaccine category, product category, the guidance states or recommends that reproductive tox studies be performed for every final clinical vaccine formulation that is used in studies that enroll pregnant women.

And in order to avoid having to perform multiple studies, the suggestion was made to really conduct phase I and II studies--of course, in non-pregnant individuals--and to only advance the most promising vaccine formulation in studies that enroll pregnant women.

Furthermore, the guidance discussed that the need to repeat a reproductive tox study for a vaccine product that is similar to a product for which a reproductive study has been done--and the example listed was the nine versus 11-valent pneumococcal conjugate vaccine--that would need to be decided on a case-by-case basis, and would depend on several criteria, such as methods of manufacture and the availability of other preclinical and clinical data.

Industry wanted clarification on how this document would be applied to therapeutic vaccines, as many therapeutic vaccines under development would be intended for use in adolescents and adults. And the guidance also does not address how it would be applied to investigational vaccines, as well as those that are already licensed.

The suggestion was made to apply reproductive toxicity assessments to those new vaccine candidates only for which the natural history and epidemiology of the "Y"-type disease would suggest untold effects on females of reproductive age, abiogenesis, and newborn development.

Industry wanted clarification on the type of changes made to the product that would require additional studies. And the point that was made was that several changes are made to the product during clinical development, and therefore not all of them should require additional preclinical studies.

We were also asked to clarify whether all vaccine formulations would need to undergo developmental toxic testing. Often, pivotal studies are conducted with the final formulation, but subsequent optimization and formulations are made, and the need for additional preclinical trials in such cases should be evaluated on a case-by-case basis.

Also, industry felt that combination vaccines under development that are composed of antigens that are already included in licensed vaccines should really not be subject to requirements for reproductive toxicity studies.

And one of the last points made in the guidance was that reproductive toxic studies should be conducted for vaccines that are indicated for adolescents and adults and for vaccines which are indicated or may have the potential to be indicated for immunization of pregnant women, but--
[Tape Change.]

1B

DR. GRUBER: --that is specifically indicated for immunization of pregnant women would need to be available prior to the initiation of any clinical trial that would enroll pregnant women. But if you have a vaccine that is indicated for adolescents and adults, it may be okay to include women of childbearing potential into clinical trials without reproductive toxic studies, provided that appropriate precautions are taken, such as pregnancy testing and use of birth control.

And for vaccines for these types of target populations, data from reproductive tox studies could be conducted as late as post-pivotal trial or concurrently with the pivotal trial. And then the data should be submitted with the biologics license application.

Industry said that the guidance needs to more explicitly address the target population to which the guidance would apply. The comment was made that the many vaccines already licensed or under development for children less than five years of age should not be subject to the guidance.

And also, the guidelines would cover vaccines that are intended for maternal immunization, as well as unintentional exposure, but the read-out and follow-up of the offspring could be expected to be different in both situations. And this should be recognized in the guideline.

And I think it may be worth spending a few minutes discussing if the read-outs for these different populations--let's say, maternal immunization, or unintentional exposure--are indeed different; especially when you consider vaccination programs that are currently being discussed that target immunization of women of reproductive age with the intent to prevent perinatal infectious disease in the offspring when the woman gets pregnant or is pregnant.

And lastly, there was a request for clarifying administrative procedures.

Now, the guidance for industry document also discussed or recommended that pregnancy registries should be conducted. And we received actually very positive comments from industry. But since it's not the scope of today's discussion, I am going to be skipping this.

And I would like to conclude this overview of the guidance and comments received from industry with questions that I think we should try to address this afternoon. And I formulated these questions based on the comments and concerns that we received from industry, and also comments and concerns that were raised in looking at data from reproductive toxicity studies and that we had in discussion with sponsors.

And in random order, the questions are:

In addition to endpoints outlined--and you have them in your background package--in addition to endpoints outlined in the ICHS5A document, what additional parameters should be evaluated? Thinking of immunological parameters, histopathology, functional assessment. Can you think of more?

If you focus on immunological parameters, what should be focused on? What should be assessed? Are antibodies enough? Do we need to look at cell-mediated immune responses, cytokines? And how far should we even assess

potential interaction with fetal tissues? Should there be kinetic assessments?

What is the extent of assessments in the dam versus fetus versus newborn? And should we consider a tiered testing approach that was suggested by industry?

How should we assess the potential for developmental immunotoxicology, given the species-specific differences in immune system maturation, species-specific differences in the maternal cross-placental antibody transfer, and perhaps species-specific immune responses in general?

Should reproductive tox assessments remain essentially restricted to pre- and postnatal developmental studies? That is, should there be no fertility and post-weaning assessments?

What parameters should be used to assess pre-weaning development? Looking at body weight, functional assessments, other issues?

How do we deal with the dosing?

How do we choose the immunization interval, keeping in mind the relationship of dosing to developmental time lines?

And should developmental tox studies differ in terms of read-outs and follow-up depending on the vaccine's indication; that is, maternal immunization, versus an indication for adolescents and adults that includes females of reproductive age?

And finally, what constitutes a relevant animal model?

What factors should go into the equation in terms of deciding what a relevant animal model is? Should we only look at antibody response? Do we need to consider other issues?

How do we deal with species-specific factors, the use of non-traditional species, the availability of background data, and the practicability and availability of species? And what alternate methods do we have available to us to assess and predict human risk if a relevant animal model is not available?

And finally, should reproductive tox assessments be required for vaccines that belong to a product class for which a large body of clinical data exists?

And that would conclude my overview of the guidelines. And we have scheduled discussions this afternoon. And we basically did a somewhat arbitrary division, where we said, okay, we're going to start discussing study designs for developmental tox studies; we're going to look at immunotoxicity endpoints; developmental endpoints; and we wanted to finish with animal models.

But we realize that there is probably going to be a big overlap, and that one issue can probably not be discussed without the other. And so when we discuss this this afternoon, I think we need to keep this in mind.

What I would like to do this afternoon is really put up these questions again. I realize we may not be able to answer them all, but indeed if we want to revise the guidance, we need to try to reach consensus on some of these issues that I have discussed this morning.

So it is 9:30 right now. I think right now we are right on schedule. If there are no pressing issues that require clarification of my talk--again, I said that we need to really discuss the issues this afternoon--I can introduce the next speaker. If not, I can allow one or two questions.

[No Response.]

DR. GRUBER: Good. So I guess my presentation was sufficiently clear.

[Applause.]

DR. GRUBER: Well, it is a great honor to introduce to you the next speaker. It's Dr. Richard Insel, who is the Director of the Center for Human Genetics and Molecular Pediatric Diseases in the AAB Institute of Biomedical Sciences. And Dr. Insel is the professor of pediatrics and microbiology and immunology at the University of Rochester School of Medicine.

His early research focused on the development and immunogenicity of haemophilus influenza B conjugate vaccines. And he was part of the research team that developed conjugate vaccines for infants, which of course,

as you know, have eradicated infant bacterial disease from invasive haemophilus influenza and eliminated the most common cause of meningitis in children in the United States.

Together with David Smith and Porter Anderson, Dr. Insel was the scientific founder of Praxis Biologics, the company that first developed haemophilus conjugate vaccine for infants. And Dr. Insel has studied the use of vaccines during the third trimester of human pregnancy.

His current research focuses on the genetic regulation of the generation of B lymphocytes, memory B cells, and plasma cells. And he is investigating the network of protein pathways that regulate human lymphocyte development and differentiation.

Ladies and gentlemen, Dr. Insel.

[Applause.]

HUMAN T AND B CELL DEVELOPMENT

PRESENTER: RICHARD INSEL, PH.D.

UNIV. OF ROCHESTER, SCHOOL OF MEDICINE

DR. INSEL: Marion, thank you.

We're going to change directions a little bit here this morning in this next talk. What I'm going to do is I'm going to provide a relatively simple overview of how the immune system develops. I'll then discuss the ages at which development occurs in the human fetus. We'll look at the maternal contributions to immunity in utero. And I

want to just provide some brief glimpses of evidence that the fetus can make an active immune response.

What the first slide shows is, I like to think of the immune system as composed of two really major components: what we call "innate immunity," and adaptive immunity.

And innate immunity exists to immediately and quickly recognize that the host has been invaded, that there is a danger on board, there's something foreign on board; and responds quickly to that response with either a cellular response, as shown here with, in this case, antigen presenting cells, APC's--one example of which would be professional dendritic cells. And they respond to contain that insult, and will invoke an inflammatory response to contain and destroy that insult.

And in addition, the innate immune system will capture this antigen and present this antigen to what we call the "adaptive immune system." The adaptive immune system is made up also of cells and proteins. And the major components, as all of you know, are lymphocytes. And they are the T lymphocytes and B lymphocytes.

Lymphocytes can generate an antigen-specific immune response which is high in specificity. It may be delayed, in contrast to innate immunity. And with that immune response, we generate an effective response composed of a cellular response; or a soluble response in the case of antibody, the product of B lymphocytes, to eliminate and

bind to that antigen, eliminate that antigen. And in addition, we induce memory, to remember that encounter in case of future exposure to that particular antigen.

Now, lymphocytes--these T lymphocytes and B lymphocytes--as is true of 11 different other lineages, all derive from a hematopoietic stem cell. This stem cell is potent, and it has regenerative capacities, and exists in adults in the bone marrow.

This stem cell gives rise to either a common myeloid progenitor or a common lymphoid progenitor. The common myeloid progenitor gives rise to seven different cell lineages. The common lymphoid progenitor gives rise to B lymphocytes; T lymphocytes; NK, or natural killer cells; or dendritic cells.

Now, it's a little bit more complicated than this. What we have is we have our hematopoietic stem cell, giving rise to our lymphoid progenitor here, and either giving rise to B lymphocytes on the left-hand side, or T lymphocytes on the right-hand side.

And lymphocyte development occurs in a very well-defined pathway, with discrete stages of development or differentiation. These stages are characterized by changes in cell surface markers, and changes in gene expression.

So on the left, if we look at B cell development, which in the adult is going on in the bone marrow, we have initially a progenitor B cell that gives rise to a pre or precursor B

cell that has cytoplasmic "U" [ph], that then gives rise to an immature B cell which has on its surface IgM, and then giving rise to the mature B cell which has IgM and IgD. That cell then leaves the bone marrow to move to the periphery.

All of this development in the bone marrow occurs in an antigen independent fashion. In the periphery, if that mature B cell comes in contact with antigen, and in the present of T cell help, that B cell differentiates, proliferates--and generally it's an antibody-secreting plasma cell--and can isotope switch to become an IgG, IgA, or IgE B cell and plasma cell.

In addition, in the periphery that B cell can undergo somatic hypermutation, that gives rise to high-affinity antibody responses. All of this is occurring in secondary lymphoid organs in the periphery in the germinal center. Somewhere on the T cell side, on the right here as we see, we also have these individual discrete stages. T cell development, in contrast to B cell development that's going on in the bone marrow, is going on in the thymus. And what we have is T cells passing through well-defined stages of progenitor T cells; precursor or pre T cells; to become an immature T cell which expresses double-positive, CD4 positive, CD8 positive T cells; to give rise to a mature single-positive T cell which is either CD8 or CD4, which leaves the thymus and moves to the periphery.

So in a very simple way, this is how development occurs, either in the bone marrow for B cell development, or in the thymus for T cell development.

Now, with each of those stages of development, there are certain decisions that have to be made. And I'm only going to give you really some take-home messages here. What's happening as we move from this hematopoietic stem cell to this multipotent progenitor, to this common lymphoid progenitor, in this case giving rise to stages of B cell development associated with changes of surface markers--in the case of B cell development, changes of immunoglobulin, gene rearrangement--there's also changes in gene expression.

And these changes of gene expression exist to make certain major decisions. One of the first decisions that has to be made is to become a lymphoid lineage cell. And what we have here is a decision that's being made with this common lymphoid progenitor for lymphoid lineage specification. What that decision really represents is the extinguishing of multiple genes that are being expressed at extremely low levels, as well as the onset of new gene expression and up regulation of other genes being expressed. What one is doing is honing down lymphoid and turning up myeloid development.

At the next stage when it moves into the B cell stage of things, extinguishes T cell development, one has what we

call B cell lineage specification associated with onset of expression of new transcription factors.

And then last, we make finally a commitment, whether it be to B cell lineage commitment or to T cell lineage commitment, associated with expression of unique transcription factors.

And in the case of B cell development, we know that the gene PAX-5 and its product BSAP is involved in B cell lineage commitment which is associated with onset of CD19 expression and onset of VDJ rearrangement.

Thus, with these development switches, what we have is unique genes making decisions for specification, and then ultimately commitment to that particular lineage.

Now, let's just turn to some very practical things as far as when does development occur. And this slide just illustrates that we have initially hematopoiesis occurring in the human fetus outside the embryo--it's in the yolk sac outside the embryo--occurring quite early, at embryonic day 18.

Hematopoiesis then switches at approximately embryonic day 40 to the fetal liver. And we begin what we call "definitive hematopoiesis," which is characterized by enucleated red blood cells, as well as production of adult hemoglobin. We then have hematopoiesis occurring in the bone marrow at approximately 12 weeks of gestation.

Lymphocyte development does not occur with primitive hematopoiesis. It only begins with definitive hematopoiesis, beginning at approximately six weeks of age, and beginning in the fetal liver.

It will then move on from the fetal liver, as I'll show you in the next slide, to the bone marrow. So lymphocyte development begins at around six weeks of age in the fetal liver, moving into the bone marrow at approximately 12 weeks of age.

This slide also illustrates on the right contrasting the human situation to the mouse situation. And one should immediately see some interesting differences.

Mouse development occurs much later in comparison to human development, in comparison to the total length of gestation of approximately 20 days. One doesn't see fetal liver hematopoiesis or lymphoid development until about halfway through the gestation period, and one doesn't see bone marrow development until three-quarters of the way through gestation; in contrast to the earlier development in man. Now, this transition from fetal liver to bone marrow for definitive hematopoiesis as well as lymphoid development is not as simple as this. But as shown on the slide, it's really a continuum. What one has is, approximately at six weeks of age, the onset of fetal liver development, of lymphocytes and hematopoiesis, which gradually peaks at about three months of age, and then tails off and is

extinguished by approximately 30 to 35 weeks of age. It is gone by the time of birth.

The bone marrow hematopoiesis and lymphopoiesis begins at approximately three months of age, and now in its primacy is more important than fetal liver hematopoiesis or lymphoid development by five months of age. And the bone marrow will continue to be the major site of lymphopoiesis and hematopoiesis throughout the third trimester, and is the sole site of hematopoiesis and B cell development in postnatal life.

Now, the way I like to think of developmental stages in man is illustrated on this slide that was prepared by Harry Schroeder, from the University of Alabama. And what he's done here is divided up development into first, second, and third trimesters. This is for the human side of things. And as a generalization, with first trimester what's going on is we're accumulating lymphocytes. T lymphocytes are developing, B lymphocytes are developing. So we see this liver development, liver lymphopoiesis occurring, around six weeks of age, and then trailing off.

We have bone marrow development, beginning at approximately 12 weeks of age, and becoming the major site of hematopoietic stem cell development. That's where the common lymphoid progenitor will be. And it will remain the site of B cell development.

We have the thymus beginning to become developed at around six to seven weeks of age in this first trimester.

And by the end of the first trimester, we have T cells and B cells that are mature--and I'll show you some data in a second--at the end of that first trimester. And we have all the players really set up.

The second trimester is associated with really peripheralization of these cells into secondary lymphoid organs. And so we have secondary lymphoid organ organization beginning. By the end of the second trimester, we have had lymphoid organs developed. We have them populated. And we have a relatively intact immune system in the human.

The third trimester is associated with further organization of those lymphoid organs. But what we have primarily is an increase in cellularity--an increased number of cells--and some increase in diversity of the repertoire. But the immune system in man is pretty much intact by the end of that second trimester.

Now, if we walk through and we look, what we'll now do is look at B cell development, then we'll look at T cell development. So this slide just begins to summarize human B lymphocyte development.

So as I mentioned, at six weeks of age in the fetal liver we have hematopoietic stem cells. At approximately one to two weeks later, we begin to see B cell precursors, these

progenitor B cells and these pre or precursor B cells now appearing in the fetal liver.

Approximately two weeks after that, we begin to see IgM positive B cells. And at about two weeks after that, those IgM positive B cells, which are considered immature B cells, now acquire IgD. So they're mature IgM positive, IgD positive mature B cells. We now see IgG positive B cells. And the ratio of progenitor and precursor B cells to B cells is approximately two to one.

If one cultures those fetal liver B cells, they can function, and they can be activated to secrete immunoglobulin. And one is beginning to see at the end of that first trimester peripheralization of those fetal liver B cells to the rest of the body.

And one then sees at that time bone marrow development, where we're seeing now hematopoietic stem cells in the bone marrow--or presumably, hematopoietic stem cells in the bone marrow. It's very difficult to identify hematopoietic stem cells. And we're seeing both pre B cells and B cells now developing in the bone marrow. And the bone marrow is becoming that site.

In the second trimester, by 15 weeks, the percentage of B cells in the spleen, lymph nodes, and blood is equal to what we see in postnatal life. And so you can see how this is very early in the development we've acquired now numbers very similar to what is happening in postnatal development.

At 18 to 20 weeks, we see primary follicles in secondary lymphoid organs, such as lymph nodes in the intestine. A few weeks later, we see primary follicles in the spleen. And then what we see in the third trimester is loss of lymphopoiesis in the fetal liver, and the bone marrow becomes the primary site.

So that's B cell development. Let's take a look at T cell development. The thymus forms at approximately six weeks from contributions from the third pharyngeal [ph] pouch, branchioblast [ph], as well as neurocrest [ph] elements. We see thymic precursors, progenitors, populating that thymus initially at approximately seven weeks. Those cells can initially be seen in the fetal liver at seven weeks, and they begin to repopulate in small numbers the thymus at about that time.

Population increases as the thymus becomes more vascularized at about eight weeks. And by ten weeks, one can see real thymic organization, where the thymus can be discerned into a cortical region as well as a medullary region with true demarcation.

By 12 weeks of age, at the end of that first trimester, we have double-positive, CD4 positive, CD8 positive, receptor bearing thymocytes. They are functional. They can proliferate to either foreign cells in an allogeneic reaction, and they can proliferate to mitogens, such as phytohemagglutinin [ph].

And by 14 weeks, we're seeing Hassels [ph] corpuscles form. And by 15 weeks, the subsets in the thymus now are very similar to what we find in the newborn. The T cells begin to emigrate to the periphery, and begin to localize in the spleen. So very similar to what we saw with B cell development, by 15 weeks we're seeing this marked peripheralization. So this is early on in that second trimester.

At 24 weeks, near close to the end of the second trimester, if one looks at the repertoire, based on looking at cord blood of prematurely born infants, one finds that the V-Beta family usage--this V-Beta is one of the genes that encodes one of the T cell receptors that's encoded by the Alpha and Beta chain--one finds that the diversity of V-Beta usage is identical--as far as proportion of V-Beta families being used, is very similar to what's used in the adult.

The CBR3 [ph] size is skewed. And that's because of the lack or the paucity of [inaudible] addition, due to a lack or low levels of the enzyme TDT, terminal deoxynucleotidyl transferase. But the bottom line is, we have a fairly diverse repertoire, even at the end of that second trimester.

And the third trimester is associated with increased cellularity in the thymus. We see some increased diversification, with increased CBR3 size. And we see

increasing cells in the periphery. So the third trimester is primarily associated with expanding those cells that are there at that second trimester.

Now, if one looks at the major peripheral lymphoid organ, the spleen, one finds by seven to eight weeks one can begin to see a spleen, and one can begin to see a few lymphocytes there. And by 15 weeks, one has in that spleen T cells, B cells, as well as IgM plasma cells.

At 16 weeks, one can see T cells localizing in what we call the periarterial lymphoid sheath, which is a correct localization for T cells. A week later, you can see follicular dendritic cells; a few weeks later after that, IgG plasma cells. And then one can see at the end of that second trimester primitive B cell follicles with follicular dendritic cells. So all the organization is there by the end of that second trimester.

Mature follicles are seen at 30 weeks. But one does not see germinal centers until after birth. And that's because one needs exposure to the outside world with activation of the innate immune response to get germinal center development.

So we've just looked at cellular contribution in the fetus to immune development. What I want to turn to now is, as all of you appreciate, the fetus also is bestowed with maternal immunoglobulin. Of the isotypes, IgG is the only isotype that crosses the placenta. Passive transport

begins in the first trimester, quite early. Active transport begins in the second trimester, and it picks up in activity near the end of that second trimester.

A prematurely born infant who is born at 30 weeks gestation will have an IgG level of approximately half of a full-term-level infant. And a full-term newborn will have an IgG level greater than maternal levels of IgG, because of this active transport.

Although all IgG isotype subclasses can cross the placenta, IgG1 preferentially is transported. And thus, when one looks at full-term infants often the level of IgG1 is higher in the newborn compared to the mother. The IgG2 subclass is not transported as well. IgG3 and IgG4 are intermediate, between IgG1 and IgG2, and being transported.

Now, with transport of immunoglobulin, one has to ask: What are the consequences of maternal antibody? Can that affect the response of the newborn or infant to immunization? And as all of you appreciate, we know that maternal antibody can inhibit replication of live viral vaccines. And this has been shown with measles viral vaccine. This is not the sole reason that infants respond poorly to measles vaccine administered in the first year of life.

But even with killed antigens, we know that maternal antibody can decrease active antibody responses of the infant to immunization with killed antigen vaccines.

This may work through one of several mechanisms, such as redirecting antibody, redirecting antigen away from antigen presenting cells. Antibody may alter antigen processing and presentation by the antigen presenting cell. And one can also inhibit B cell responses secondary to antigen antibody complexes which can send an inhibitory signal to the B cells through co-stimulation of surface immunoglobulin and the FC gamma R2 [ph] on the B cell surface. So antibodies from the mother may suppress infant antibody responses. And one must keep that in mind.

In addition, one also has to appreciate a subject that was discussed yesterday by Dr. Lambert: the possibility of auto-antibody production. And we know that with transfer of immunoglobulins across the placenta, if the mother has auto-antibodies those may be transported across the placenta to the fetus, and may give rise to symptomatic disease. Thus, mothers with lupus who have anti-ro [ph] and anti-la [ph] antibodies, their infants may have either a neonatal heart block, or cardiac endofibromatosis [ph] may occur with their hearts.

Obviously, Rh incompatibility, ABO incompatibility, antibodies to platelets, can give rise to thrombocytopenia, and antibodies to white cells can give rise to leukopenia-- very well known reactions. And newborns born to mothers with myasthenia gravis or thyroid disease may also develop those diseases, such as myasthenia or thyroid toxicosis.

And maternal antibodies can also cause membranous glomerulonephritis in the offspring. So it is something that we must also keep in mind.

Now, in addition to maternal antibody, what about the fetus? Is the fetus capable of generating an antibody response? And the answer is "Yes." If one looks at cord blood, one finds a level of IgM, which we know doesn't cross the placenta. That level of IgM is approximately 10 percent of the adult level.

We know that that immunoglobulin may be associated with antibodies to blood group antigens such as blood group "I," blood group "A," or blood group "B." And we know that this is a fetal contribution, because one can identify paternal genetic markers, or paternal allotypes, on that immunoglobulin.

In general, these IgM antibodies are low affinity. They are poly-reactive. They have not undergone a somatic type of mutation. They're germ-line encoded. And we know that antibody production can occur as early as the second trimester.

Now, I just want to point out, there are three, I think, pretty good examples in which we have documented evidence that the fetus can make an immune response. They can be found associated with congenital infections in the fetus; where the fetus has been in utero in an environment where

the mother has had either a parasitic infection or an infestation, or with allergen exposure.

So we know with congenital infections that the fetus can generate an IgM antibody response. With CMV, about 90 percent of offspring will have an antibody, if they have congenital CMV. With toxoplasmosis, it's about 81 percent. With rubella, it's approximately 65.

And it's not solely IgM antibody. If one looks at IgA antibodies, we know with toxoplasmosis up to 89 percent of fetuses will have an IgA antibody response to toxoplasmosis.

If one looks early on in gestation, at prematurely born infants, newborns born with congenital toxoplasmosis, one can find antibody responses in a quarter to a half of those newborns. Thus, antibody production is beginning quite early in life with these congenital infections.

Over the last decade it's been shown that parasitic infections can activate immune responses in utero, and can prime for immune responses. And this has been shown with schistosoma mansoni [ph], with trypanosoma cruzi [ph], with plasmodium falciparum [ph], with helminths.

And if one looks at cord blood, one can culture cord blood lymphocytes--and specifically cord blood T-lymphocytes--with antigens from these parasites, and show specific T cell proliferation. One can show that those T cells not only proliferate, but will produce cytokines. And they

will produce cytokines, not just TH1 cytokines; but will produce both TH1 as well as TH2 cytokines.

You can demonstrate a specific IgM antibody response in cord blood to those parasitic antigens specifically in offspring of infected women. And one can also culture newborn B cells and demonstrate in vitro an IgG antibody response to parasitic antigens.

Last, with allergens, both with indoor as well as outdoor allergens one can demonstrate, using cord blood lymphocytes, T cell proliferation. One can demonstrate proliferation of not just naive, but memory T cells. And one can demonstrate that those T cells can make multiple cytokines, often of the TH2 variety, IL4, IL5, IL10, and IL13. And one can generate even allergen-specific T cell clones, and show that they have the ability to generate these cytokines.

Thus, congenital infections, parasitic infections in the mother, as well as allergen exposure, all appear to be able to prime responses in the fetus.

Now, the subject of maternal immunization has arisen, And I just want to point out that with maternal immunization-- for example, with Group B streptococcal vaccine that's being currently studied, as Marion just pointed out--the mother can generate a serum IgG antibody response. And that IgG can be transported across the placenta to the fetus.

Two comments about that. One, one has to realize that there will be a lag in antibody production, which is true no matter if you were immunizing a pregnant or non-pregnant woman. And there's also a lag in transport.

If one immunizes late in gestation, at approximately 38 weeks of gestation, one will not find elevated levels of antibody in the offspring. One has to immunize early, to allow the FC receptors in the placenta to become saturated with the antibody and then transport it actively across the placenta.

And thus, for Group B streptococcal immunization during pregnancy, those immunizations are occurring at approximately 32 to 34 weeks of gestation, to give enough time for an active antibody response of the mother, as well as time for transport of that antibody across the placenta to the offspring.

In addition to making IgG antibody to the vaccine antigen, there is the theoretical possibility that the mother could make an IgG anti-idiotypic [ph] antibody to that antibody to the vaccine antigen that could conceivably act as a mimic of the vaccine antigen. That is something that has been documented quite well in animal models, but not documented very well on the human side.

There is the theoretical possibility that the vaccine antigen itself could cross the placenta. But there is not very good data showing that that occurs in man.

And last, another contribution from maternal immunization is from breast milk antibody. It's well appreciated that if one immunizes during pregnancy or after pregnancy in a lactating woman, one can increase levels of antibodies, specific antibodies, in colostrum or in breast milk.

And these are studies done almost two decades ago of women who were immunized in the third trimester of pregnancy with the haemophilus influenza polysaccharide vaccine. And they had levels approximately 20-fold higher in their colostrum, compared to non-immunized women. And levels were quite elevated as well in their breast milk, compared to non-immunized women. And this has been shown for many other kinds of vaccines.

Now, one of the questions that one has to struggle with: What is the evidence, are neonatal B cells activated or primed during maternal immunization during pregnancy? I mentioned that congenital infections, as well as parasitic infection in the mother, as well as allergen exposure, can prime in utero. How about active immunization during pregnancy?

Well, the bottom line is that on the human side there's not a lot of data to suggest that this is occurring. When looked at for haemophilus influenza B, influenza virus, at Group B streptococcus, at pneumococcus, there is no good evidence that it occurs.

However, for tetanus there does exist some data--initially, from Tom Gill's [ph] group at Pittsburgh--suggesting that tetanus immunization earlier in pregnancy, earlier than the third trimester, as well as possibly multiple doses of tetanus immunization in other studies, may have the ability to prime the fetus for an IgM response to tetanus.

More recent studies though have not validated this; although I need to point out that those recent studies were done during the third trimester of pregnancy. This is something that really does deserve further study. And it's probably the type of study that should and could be done in the developing world. And I urge that that be further looked at.

Last, in closing, I just want to point out, if one looks at the neonatal immune system, just a couple of generalizations. If we look at the immune system of a full-term infant, what do we have? What we have is, we have an intact immune system, but it's a naive immune system that just has not been primed yet. So we have this, what we say, immaturity, but it's immaturity due to lack of antigen exposure.

And I think it's important to point out that the human neonatal immune system is far more mature than the murine immune system. The second-trimester human fetus is comparable to the newborn mouse.

And I think one can appreciate why that is if one looks again at some of these numbers. What one finds is, as I pointed out earlier, if you look at mouse development, one is not seeing fetal liver development, fetal liver B cells in the mouse, until about day 14, and bone marrow hematopoiesis and lymphopoiesis until about day 15, in this 20-day gestation period.

In contrast, as I pointed out, by the end of the first trimester we have fetal liver development, bone marrow hematopoiesis and lymphopoiesis intact. And by the beginning of the second trimester, we're seeing peripheralization of these lymphocytes. Thus, the kinetics of development are quite different in the two species.

If you look at the neonate as well as the infant on the B cell side, it's just important to remember that what we do have often in the neonate, we can have a low-antibody response. Sometimes it's transient. It's lower affinity, because often it's germ-line encoded. It can be inhibited by maternal antibody. You can see a decreased germinal center reaction.

But you do activate memory B cells. And in fact, if anything, memory B cell activation seems to be less stringent than induction of primary antibody responses in the neonate and young infant.

There may be restricted repertoires early on in life. And we do have this age-related hierarchy of responses. As we

know, responses to polysaccharides don't occur until usually two years of age or later.

And similarly, on the T cell side, the newborn is not born with any kind of T cell memory. He or she has a naive repertoire. And those T cells don't proliferate and generate cytokines as well as adult cells. And they require co-stimulation, but this is because they're naive cells. And this is really a property of being really a naive cell. And what they do need is really optimal antigen presenting cell, or innate immunity adaptive T cell interactions. And that co-stimulation is very critical for naive cells.

Last, in closing, there was this belief that the newborn could only generate a TH2 response. And we know that the newborn can generate a TH1 type response. And a good example of this is the work of Arnaud Marchand [ph] and others, in looking at the responses of newborns to BCG. And what he and others have demonstrated is that BCG, if given at birth, can generate a very good TH1 response with high levels of Interferon-gamma and low levels of IL4. And then, as was brought up by Paul-Henri Lambert yesterday, with that BCG immunization in the neonate or infant that generates this TH1 immune response, the BCG can increase antibody responses to hepatitis B virus, but not the tetanus or diphtheria.

But it's important to remember that in spite of a very potent immunization such as BCG that generates a TH1 response, that TH1 response in no way polarizes an immune response to other vaccines that are administered either simultaneously or later in life. So one doesn't have immune deviation, even with immunization occurring in the neonate with a potent vaccine such as BCG. And so one needs to keep that in mind.

Thus, in conclusion, what we've seen is the first trimester in man is associated with initiation of lymphopoiesis, production of T-lymphocytes and B-lymphocytes. And by the end of that first trimester, we're seeing the beginning of peripheralization from either the fetal liver or from the thymus to the periphery of B cells and T cells, respectively.

In the second trimester, we're establishing lymphoid organs that become populated by those cells. We're getting normal structure formation.

And the third trimester is primarily associated with increased cellularity, increasing the number of cells of those subsets that are there by the end of that second trimester, with some increased diversification.

As I have noted, the fetus can generate immune responses to congenital infections, to allergens, as well as to protozoan antigens. The fetus can acquire maternal IgG,

and it's something that one will have to keep in mind. And last, the human is not equivalent to the mouse.

I thank you for your attention.

[Applause.]

DR. INSEL: I'd be glad to answer any questions.

PARTICIPANT [In Audience]: I was wondering if you had information on why some antigens cross the placenta? You said that apparently with allergens or parasites, immune responses occur. So I presume that's due to the material crossing the placenta; and why others done.

DR. INSEL: Yes. It's a good question. And it's not been really well studied. I think one of the things is, with the parasitic antigens I think the level of antigen exposure is probably very important, and the chronicity of antigen exposure.

And exactly how transport is occurring, whether it's occurring bound as an antibody antigen complex that's transported, or whether it's transported separately as an antigen, has not been really well studied.

But I think the level of antigen probably is quite critical. But highly deserving of further study.

PARTICIPANT [In Audience]: I was just wondering if you could comment on anything about NK cells and development?

DR. INSEL: Yes. I can't. You know, there are different populations of NK cells. I don't really have the data.

But if you come up to me later, I'll be glad to look it up and send you that. But I can't give you good data.

MR. PARKMAN [In Audience]: Actually, this question, or this comment, is not directly related to your talk. But if people or if the organizers will forgive me, I would like to kind of comment on the whole discussion we've had here this morning.

My name is Paul Parkman [ph]. I was with the regulatory agency from 1963 until 1990, so I was there for CBER in CDB, and then CDB and all of those places.

Since I have left the organization, I have been a consultant. And just so people know where I stand, I have consulted not only with the government, but also with manufacturers, including Aventis Pasteur and Merck. Nobody has told me what I should say, I would hasten to add. So these are only my own thoughts. And I kind of worry about--

[Tape Change.]

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MR. PARKMAN [In Audience]: --one product that has had the potential for bad reproductive toxicity, and that was German measles vaccine. And I'm surprised there wasn't more mention of that. That was a long time ago, of course. It was in 1969.

The studies that were done that suggested that the vaccine was not reproductively toxic were done almost entirely by the Division of Biologic Standards. And the results in

animal models, the monkey was selected. I mean, we kind of looked back to epidemiology, and saw what the disease did. We used monkeys as a model, because they are kind of close to man. We developed a model for the disease.

We studied pregnant Rhesus monkeys. We looked at the outcomes of infected pregnant Rhesus monkeys. We made markers for the attenuated product, the attenuated vaccine. We, along with CDC, followed up after the vaccine was licensed, to look at women who were inadvertently vaccinated. And it showed that the vaccine was safe, I think most people believe.

You know, and another reason I got up is I'm kind of alarmed by rumors of a 400-rabbit toxicity test for a vaccine that was recently being considered. And maybe it isn't 400; maybe it's somewhat less than that. But that's a very large experiment.

Very large experiments take away from personnel time and effort in trying to develop new products. So I kind of come around to the point that I would encourage kind of caution in what kind of testing the agency would require. And I say all this because I know there's a lot to come today about people who will talk about toxicity testing. I thought your talk was excellent. But it also causes me to worry--because it sounds very "researchy"--as to what the FDA might require.

I would only counsel that the FDA be careful. It takes a lot of time and effort to do these studies under GMP, that can take away from what people can do on other things. I think it would be worthwhile some time to review the reproductive toxicology and the toxicology of products that have been approved before, not only rubella but perhaps other topics. If thimerosal is a big issue, maybe it would be worthwhile to look and see what is being done now to look at that issue.

And I'm sorry, I probably have gone on too long. But anyway, thank you.

DR. GRUBER: Yes, Dr. Parkman, thank you very much for your thoughtful comments. I just wanted to mention that actually the FDA is by no means there that we require reproductive tox studies in 400 rabbits.

As a matter of fact, I think today's discussion is all about how to really approach reproductive toxicity assessment in the most feasible and practical way, and I think I sort of mentioned that at the beginning of my presentation. But I think we're going to be discussing some of your concerns this afternoon, and we should keep those in mind.

I think we're just going to allow for one more question, and then we are actually entitled for a coffee break, so that we're not running too late.

MS. LINDBERG [In Audience]: Rae Lindberg [ph], SRI.

I wanted to thank you for a really wonderful talk that gives us encouragement that these studies are extremely relevant and probably feasible.

I wanted to ask you, you've convinced us that a mouse is not a man. And I wonder if you can lead us to any other small animal model? Or are we really constrained to think of primates as the appropriate model for these sorts of studies?

DR. INSEL: Yes, it's a great question. I think it's very difficult to use small animal models. And I think in certain instances one is going to need to look at primates. I think one will have to do this on a case-by-case basis. I would hate to generalize.

But I think one can learn some things from small animal models that can be relevant to the human experience. But one can't directly extrapolate for sure from mouse to man. I mean, that's going to be obvious.

Also, I urge whenever possible in the human situation to try to study man; whenever it can arise and one can get cord blood to look at, lymphocyte subsets to look at, responses. It's difficult, obviously, to get blood from infants. I appreciate that. But where inadvertent immunization has occurred, where exposures have occurred, especially with this registry, I urge people to try to look at the human situation whenever they can, so we can learn as much as possible. Thank you.

DR. GRUBER: Okay. So I think we're going to have a 15-minute coffee break. And we reconvene at 10:30.

[Recess.]

DR. GRUBER: I would like to now introduce our next speaker, and that is Dr. Stephen Holladay. He is a professor of anatomy and toxicology at the College of Veterinary Medicine at Virginia Polytechnic Institute. And his research area is developmental immunotoxicology. He has recently expanded this focus of his research to include elucidating mechanisms responsible for maternal immune protection against teratogen-induced birth defects in mice. And I welcome Dr. Holladay to this session. Dr. Holladay.

MATERNAL IMMUNE SYSTEM STIMULATION

AND EFFECTS ON FETAL TERATOGENESIS

PRESENTER: STEPHEN HOLLADAY, PH.D.,

COLLEGE OF VETERINARY MEDICINE,

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DR. HOLLADAY: Thank you, Marion. It's a pleasure to be here.

I thought after I agreed to this subject, this talk, with Marion and with Ken Hastings, and picked this title, that most of you might assume I'm talking about increased risk of teratogenesis associated with maternal immune stimulation. And this is a rather paradoxical phenomenon, to me anyway. But actually, what I'm going to talk about is

decreased risk of teratogenesis with maternal immune stimulation.

This is not a new area, in one regard. It began in 1990, and then died for a while. And we picked it up about 1998 in my lab, and have been working with it ever since, more for interest than any other reason. This is not what I do for my living--Or I suppose it's not. Just recently, we were awarded five years of funding from NIH to investigate mechanisms as to how this process works. So now it's become more of what I do for a living.

But I'm going to argue that at least in a mouse model, maternal immune stimulation has what appears to be broad-spectrum efficacy for reducing birth defects caused by a number of teratogens.

I don't know if this phenomenon works beyond a mouse model. I would like for the audience to consider today possible human cohorts, as we look through this data, where we might test the hypothesis that a similar mechanism is operating in humans but has not been recognized yet.

The beginning of this concept was in 1990. Note the journal, the "Journal of Experimental Medicine." The group that published in this journal was a Japanese group, primarily involved in cancer research. The head investigator of that lab's name was Nomura. Why they shifted into teratogenesis for a brief time, I'm not sure. It wasn't talked about in the paper. But their data were

very interesting, and this caught our attention, actually about 1991, when we first saw these data.

Very briefly, these individuals used an immune stimulant. The stimulant is Pyran copolymer. And some of you that work in oncology may recognize that this was used maybe 15 or even 20 years ago to stimulate the immune system of individuals with cancer. The idea was then that activated immune cells would find and eliminate pre-cancerous or cancerous cells, and this might be therapeutic for the cancer. It proved that it really wasn't of much value, so it's not been used in that regard.

These individuals used Pyran copolymer in a mouse model. The mouse model was an ICR, or a CD mouse, basically. These "I's" indicate it's an inbred ICR mouse model. That should be an "I" also. I'm not sure how that "O" got there. But this is an imported table, and a little difficult to change. So their mouse model was an inbred ICR mouse model.

The immune stimulation was an IP injection of Pyran copolymer on the third day of gestation, and then subsequently these mice were challenged with various teratogens.

And you can see in this case that these are not all the data from their paper. The paper was quite rigorous. Many replicates were indicated. Again, the journal is a good journal, with a powerful peer review process. We had to

assume that this was a well done report. And indeed, when we validated in our own lab, we found the same results. But briefly, the first teratogen they discussed was urethane, or ethyl carbamate. This is an agent we used to use in biology labs. I can remember when we would anesthetize frogs in the lab, and reach in and pull the frog out for dissection. It's not used that way any more, because we recognize now that urethane is a carcinogen. But it also is a teratogen, and on day nine of gestation in the mouse will produce digit defects, and on day ten will produce cleft palate. With this very simple form of immune stimulation, the Pyran copolymer is an inert substance; it's sterile. But the resident macrophages recognize this as a foreign particle, and will activate and phagocytize it. And this is a very simple immune activation procedure. And for some reason--this is what I described as paradoxical--if this immune stimulation is performed, we have a reduction in the number of fetuses that have birth defects, from 25 percent of the fetuses to 6 percent. Very dramatic; four-fold reduction in birth defects, caused by that immune stimulation procedure.

A second chemical they evaluated was methyl nitrous urea, which is an alkalating agent. And in this case, digit defects were produced.

And the birth defects were reduced by the Pyran copolymer immunization from 35 to 20 percent, approaching a two-fold

reduction. A physical agent was also investigated, X-rays. Tail defects were the predominant defect. And we see here a two-fold reduction in that defect.

So when I was first called in to examine this paper, my feelings were it's kind of hard to imagine that this really works. But I know that the paper underwent rigorous review, and I know these investigators are a strong laboratory. So I recommended that we evaluate it in our laboratory as well and see if we got the same results. It could be quickly done.

We actually had a colony of C57 females we would breed with C3H males. This produces the hybrid B6C3F1 offspring that is the immunotox testing mouse used by the NTP to produce the currently most accepted risk assessment paradigm. So we had these mice in-house and we could use them. This is an inbred line. Both of those are cytogenetic lines, and this is a hybrid of that line.

And our initial experiment with methyl nitrous urea you can see here, with dosing the same level as in the paper I just showed. We produced about 56 or so percent defects. If we immune activated with Pyran copolymer on day three of gestation, which is about six days before the teratogen challenge, we have a significant decrease--about one-third--in the level of digit defects caused by this teratogen.

In this experiment, the first experiment, we had enough animals that we could use a vehicle exposed control. In

subsequent experiments, we've used immune stimulated controls. The immune stimulation has not produced undesired effects on the pregnancy. In fact, it appears to have some desired effects--decreased resorptions, and so forth--in addition to the reduced teratogenesis.

This particular experiment was the only one that I've conducted where a vehicle exposed control had a spontaneous defect. That's why there's a little bit of height on that column there. We had one exencephaly in that experiment. These inbred mice are a bit harder to breed and a bit more expensive to breed than outbred mice. So our next question was: What happens if we do this in an outbred animal?

And these are ICR--Again, a CD1 mouse, an outbred mouse. And we repeated the same experiment: methyl nitrous urea, Pyran copolymers, the immune stimulation given, IP. And you see a similar profile here, in terms of reduction of the birth defect, the digit defects.

The noteworthy difference--and we've seen this repeatedly in experiments between inbred and outbred animals--is the outbred animal tends to have a lower level of defect; in this case, a bit over 20 percent, compared to approaching 60 percent on this side.

And the outbred animal also has responded better to the immune stimulation, in terms of reducing the birth defects. Here we have about a 30-percent decrease; and here we've got more than a two-fold decrease in birth defects, digit

defects caused by this immune stimulation. So we have moved to outbred animals, and now that's primarily what we use.

We also--Well, that's a hair trigger there. Let's see.

Okay. Evaluated the same defect caused by another chemical. This is urethane. Again, we've done this with different immune stimulants, and under different conditions.

In this case, the immune stimulant is different. It's BCG, an attenuated bacillus, we used by IP injection. The same idea: To activate peritoneal macrophages.

And this was a dramatic result in this particular experiment. We had digit defects at about 19 percent, reduced to zero here in this group. The immune stimulation totally blocked the occurrence of this defect in these mice, even though urethane was given at the same dose, same schedule, and so forth, in both of these mice. The only difference was the IP injection of BCG earlier in gestation in those mice.

These peaks have a little height. I put that in there so they would be there. They technically have a height of zero, if you're wondering about that. I just didn't feel good about putting that star over nothing. So that's where that came from.

We evaluated cleft palate also by urethane, and probably have spent most of our time there, in terms of trying to

understand mechanisms by which this immune protection might work.

This is, again, in an ICR mouse model on top. You can see the normally-formed palate in this mouse. Here's the nose of the mouse; brain stem back here; the lower jaw has been removed.

We found early on that when we dose with urethane--this was at a relatively high level of about 1,000 milligrams-per-kilogram--on the morning of day ten of gestation, we could create cleft palate in about two-thirds of the fetal mice. Also, we noted that the cleft palate we produced was of two phenotypes, without much of an integrate in between. We have what we called a "wide cleft." I hope you can see that from back in the seating. And we have what we called a "narrow cleft," a more slit-like cleft. And it is probably something we can explain fairly readily by precise timing of closure of the palate with the chemical exposure, but we did have these two very different phenotypes. And we characterized them with the immune stimulation, as well. Different stimulants were being used in the lab. I tried to get away from BCG because, while it was very effective for us and worked well, contamination of laboratory personnel might result in a positive TB test, and we don't need that. Actually, I switched to BCG because I ran out of Pyran copolymer; contacted the Hercules Corporation that produces that, and they indicated, "You know, we stopped

over ten years ago. And what we've been supplying has been on our shelf, and that's gone now." So we actually had to switch immune stimulants, and that was probably good for us.

But we asked the question of: Why not Interferon-gamma? This is a macrophage activating protein. And the literature is suggesting that macrophages are role players in this phenomenon, and that their activation is very important. So why not just inject IP Interferon-gamma? So that's what we did in this model of urethane-induced cleft palate.

And we also wanted to know: If we did a more remote immune stimulation, what would happen in that case? There were other reasons to suspect this might be worth looking at. But we used a foot pad injection of a low level of Freund's complete adjuvant, and then evaluated cleft palates.

In this urethane-exposed model with these two immune stimulants, total cleft palate, you'll see, was about two-thirds of the animals in the urethane-exposed group. These divided into the phenotypes I just showed. They were predominantly the wide cleft. About 86 percent of the clefts we saw were wide clefts; about 14 percent narrow clefts. And you can see how the immune stimulation changed that profile.

Interferon-gamma injection reduced to about 46 percent the cleft palate incidence. And then, of those clefts that we

had, only 45 percent, rather than 85, were what we considered the more severe, or the wide cleft palates. So there's a change in two directions here.

With Freund's complete adjuvant the data are very similar. Again, instead of an IP injection, this is a foot pad injection at a remote site; a different form of immune stimulation. Yet the data are quite similar. We have the same reduction in cleft palate, a very similar profile of shift between the narrow clefts and the wide clefts in this model.

I have a graduate student now who is using the same immune stimulants, but in quite a different model. His interest is diabetes. This is insulin-dependent diabetes mellitus, which we know increases risk of birth defects in humans. There are mouse models for studying mechanisms behind the hyperglycemia and the associated birth defects.

And he took advantage of this system, and induced three levels of blood glucose by using a streptozosin [ph] induced diabetes. This is a longer hensible [ph] toxicant. And he produced what he called a low and a moderate and a high blood glucose group, and then focused on this high blood glucose group, which you see down here.

Abnormal to live: These are malformed fetuses. Fifty percent of the fetuses were malformed in this high blood glucose group. Those were predominantly exencephalies caused in this case. There were a few cleft palates and a

few other defects, but the majority of these defects are exencephalies.

And you'll see with the immune stimulants--this again is complete Freund's adjuvant--this was reduced to 21 percent. With GMCSF, a colony stimulating factor, this was reduced 23 percent. And Interferon-gamma, again, 14 percent.

There is no significant difference between any of these three. All three are significantly below the 50 percent in this case. So the immune stimulation again worked approximately equally, and in a very different model, for reducing birth defects.

This student noted that placental weight was significantly increased with Interferon-gamma injection, and had an interest in the possibility that the placenta was important also in this protection. And I'll show some slides along those lines in a bit.

But now this slide summarizes data currently available in the literature that demonstrates that maternal immune stimulation in a mouse model reduces chemical or other teratogen-induced birth defects.

This is in a paper in "International Immunopharmacology," a review paper we just published a few months ago. And if you have any interest in that, you can just search on my name, and this will come up. The reason I put it in is to show how diverse the teratogens are that have been used with this procedure.

Here is TCDD, or dioxin, that produces cleft palate when given on day ten of gestation.

This is cyclophosphamide that produces craniofacial or limb defects.

Urethane, we've talked about: hyperthermia; produces exencephaly.

Diabetes mellitus, I've mentioned.

Methyl nitrous urea.

Valproic [ph] acid. I had a visiting scientist in the lab interested in valproic acid. She injected mice to produce exencephaly with this drug. This is the anti-seizure drug, sodium valproate, used for epilepsy; and does increase risk of neural tube defect. And reduced this defect with a Freund's complete adjuvant immune stimulation, from 53 percent down to zero. Again, the defects totally went away in this case.

A number of these mice without immune stimulation were born with open eyes, and mice normally are born with closed eyes. And we noted that that also was significantly reduced.

This was an interesting experiment for another reason.

This is the first case where we saw a defect that was apparently caused by the immune stimulation. Mice that were exposed to sodium valproate and also Freund's complete adjuvant, a significant number of these mice were born without tails. That's not typical for sodium valproate.

That's not a defect associated with this drug. It is very rare in the ICR mouse model we use, an anuria defect. So we're presuming--We've only done this experiment once, actually. This is, I think, the only one up here that's non-replicated. But we did see an increase in anuria, or tail-less mice, in this case, which was kind of interesting. X-rays, again, here, also.

So diverse teratogens. The immune stimulation procedure can be quite diverse. Some of these I've talked about. These investigators injected rats' splenocytes. This would be an allogeneic--or actually, a xenogeneic cell in a mouse model, which would induce an immune response.

And I think we've seen all these other immune stimulants in earlier slides.

Defects, again, that are protected against are of a variety. Here's the level of birth defects without immune stimulation; with immune stimulation. And you can see in all cases we have a significant reduction in these defects. So it's a broad-spectrum thing.

The question that immediately comes to mind is: What's the mechanism? How does this work? And I'm going to tell you now, I don't really have the answer to that. But in recent--well, in the last year and a half, this is the area we've been focusing in.

The earlier report in 1990 suggested that the mechanism might involve activated immune cells that cross the

placenta and find and eliminate pre-teratogenic cells. And they actually presented what I would say is limited data. And they readily admitted that this might not be the operating mechanism. It wasn't oversold by any means, but simply suggested.

And our laboratory had questions about the possibility that this was occurring, and that part of the fundamentals of reproductive immunology is that maternal immune cells don't routinely traffic across the placenta. There is low-level trafficking of some cells; for instance, NK cells. But when placental barriers break down to maternal immune cells we see pathology in the fetus in the form of a graft[ph]-versus-host response. So we really didn't believe this was the case for the immune protection that we were seeing. This hypothesis also came out of a cancer lab; and again, with Pyran copolymer. I could reread this to sound like the cancer hypothesis, where activated immune cells find and eliminate pre-cancerous cells. So it's kind of the same hypothesis, restated for a developmental scenario. Other reasons we didn't think that was going on: Pre-teratogenic cells in a fetus are going to be semi-allogeneic relative to the mother. And it's difficult to understand how the maternal immune system might separate those from other fetal cells.

But beyond that, for some of these chemical agents--and dioxin is a good example--the defect, the cleft palate

defect in this case, is associated with a failure of apoptosis of cells lining the palatal shelves. This is an event required prior to proliferation of the underlying mesodermal cells that will then cause closure of the palate.

If these epithelial cells fail to respond to death signal and apoptose, we have to consider that the pre-teratogenic cell in this case is actually a phenotypically normal cell that didn't die. That raises further questions about: If maternal immune surveillance in the fetus is causing this effect, how are these immune cells recruited into the fetus, and how are they recognizing these phenotypically normal cells as different from other cells? So we had a number of questions about how that might work.

And our thought was that this is not a direct effect; it's an indirect effect. The likely mediators are cytokines. There are considerable cytokines that might be investigated.

Oh, we've lost part of that slide. Okay, well, that's okay. I wasn't real fond of that slide, anyway.

[Laughter.]

DR. HOLLADAY: We did perform a cell tracking study to see if we could track cells across the placenta, activated immune cells from mother to fetus, using a probe, the chloromethyl dichlorofluorocene diacetate--quite bright on

flow cytometer. And the gist of that site was, we couldn't do it.

So turning to possible mediators of this effect, we were interested in cytokines. Our immediate dilemma was that activation of the macrophage causes production of more than 100 described proteins. And these proteins in turn operate on other cell types to cause secretion of even more proteins. So our enthusiasm was diminished for trying to sort through the number of proteins we would have to, to find the active ones; which are in all likelihood acting in concert with each other, several proteins as a family, rather than one or two, anyway.

So our thought was, if cytokines are the mediators and are crossing the placenta, then there are placental targets, or there are fetal targets, that we should be able to show a change in. And these are gene expression targets.

The literature is very poor regarding ability of cytokines to cross the placenta, we found out right away searching. Interferon-alpha is described as crossing. TGF-beta is described as crossing placenta, and in a mouse that's an important cytokine development.

CSF1 crosses placenta very readily. GCSF1, granular cicolomine [ph] stimulating factor, crosses placenta. I would like to know if GMCSF crosses. I can't find that type information.

But our presumption was that if these cytokines are regulatory molecules and are crossing the placenta and operating in the fetus, we should be able to see changes in gene expression. There are focus arrays available now to do what we wanted to do then, but there weren't at the time. So we used RTPCR, and just selected a group of genes that are important in controlling cell cycle-- proliferation, differentiation, apoptosis--a few genes, and evaluated the expression of these genes.

And briefly, the expression in particular of these isoforms of BCL2 with P53 in the fetus are described as important, believed to be. And I believe they are important for controlling the balance between proliferation and differentiation.

So we examined these in target tissues. in this case, it was fetal head. The fetal palate I think would be better. And we can do that now using real-time PCR, and focus these data. But here we see that urethane reduces expression, the expression ratio of BCL2 to P53 in the direction of P53.

If we had to predict what that means, we would say that's a shift towards increased apoptosis. With immune stimulation, Freund's complete adjuvant, here you see this is normalized. Relative to control with Interferon-gamma, it's actually a bit beyond control. So this is returning gene expression in the fetus.

This is kind of a novel thing. It struck me when we saw that, that maternal immune manipulation is altering expression of very critical cell cycle controlling genes in the fetus. So we thought about the fetus for so long as a genetically pre-programmed entity that derives nutrition from the maternal organism, but other than that largely directs its own development. And these data would suggest that maternal influences might be more than we've thought. And the immune system in this case is exerting an influence on gene expression in the fetus--protein, KNAC, alpha gene. And the protein products of this gene can influence expression of both BCL2 and P53. We evaluated that. And you can see that urethane drove that expression level down. And immune stimulation with one of these, Interferon-gamma, increased it.

I'm not going to overly speculate, again, about what these mean. But analyzing the data and choosing gene ratios--in this case, which way is the best to look at it--is difficult, to say the least. I was happy at this stage we only had five genes that we were considering.

We did do a form of cluster analysis, called "principal component analysis." It allowed us to give a coordinate expression value to gene shifts with "N"; and the "N" in this case being the mother. And this would be summated gene expression for a litter of animals.

And you see in the control window here, each one of these dots represents a coordinate gene expression value for these five genes for a litter-worth of animals. The urethane is shifting this coordinate gene expression to the left and slightly up, in this graph of two principal components from the principal component analysis.

This is available in a software package--it's on the Web--from the University of Pennsylvania. I'm seeing a little bit more of this type of analysis, as we all fight with how do we evaluate expression of multiple genes simultaneously. With Freund's complete adjuvant injection, you'll see that the coordinate gene expression--these yellow squares--is shifted down, so it's normalized along this PC3 axis. With Interferon-gamma injection, it's shifted further, so it's beyond normal along PC3, and closer to normal along PC1.

And basically, this is what we saw in the preceding slide, the same information. So it's kind of a neat picture. I like the picture, again, which gives the message that maternal immune stimulation is changing gene expression in the fetus, and is in part normalizing the change caused by urethane, which we're presuming is related to teratogenesis.

So we've been developing hypotheses as to what is occurring, what underlying effects are responsible for immune protection against birth defects. One of our

hypotheses now is that immune stimulation is acting, at least in part, to restore dysregulated apoptosis.

The idea that many diverse lesions in development are caused by a similar underlying defect is not new. And that's what we're pursuing here. I suppose a good example of that are the chemicals that cause the right forelimb ectrodactyly. In other words, we're losing the lateral-most digit, or two digits. This defect can be caused--a very specific defect--by a number of pharmacokinetically and dynamically different chemicals that all seem to effect distal limb polarization.

Our hypothesis is that immune stimulation is restoring a dysregulated apoptosis. And I've tried to present some of the data from the literature that would support this.

Cyclophosphamide we know produces craniofacial defects.

These are associated with excessive apoptotic death in heads of the fetal mice. And maternal immune stimulation will reduce those defects.

Cyclophosphamide also produces distal limb defects. These have been associated, again, with increased apoptotic nuclei. Sections were cut of these limbs, and we find that maternal immune stimulation reduces those apoptotic nuclei, and also reduces the distal limb defects.

So what I'm trying to do is collect enough data that it becomes compelling. Again, our gene expression data showed that the teratogen caused a shift in the BCL2-to-P53 ratio,

that would lead us to predict increased apoptosis is involved in that defect. Immune stimulation with either of two stimulants shifted this ratio back towards BCL2, and that's a shift we would predict would be in favor of proliferation over apoptosis.

In this case we're seeing the same thing--Wonder what that check came from. It's interesting how computers communicate. We suggested a number of effector molecules that may be involved. I'm going to go by that, because they are on other slides anyway.

Some more information about potential mediators: In this case, TGF-betas that are involved, the TGF-beta-2 mRNA and TGF-beta-2 protein, found to be elevated in fetal mouse heads after injection of cyclophosphamide. Immune stimulation blocked both of these increases--this again is a gene expression effect here--blocked these increases. Interestingly enough, increased TGF-beta in proliferating fetal tissues is believed to act as a signal to cause increased cellular apoptosis, by inducing P53 gene expression. So it's again supportive of a basic argument of restoration of a dysregulated apoptosis.

Cyclophosphamide also increases TNF-alpha expression in fetal heads. Maternal immune stimulation will reduce the defects associated with that, and it also increases this TNF-alpha mRNA, or the transcripts in the head and brain of the fetuses.

And interestingly enough, again, TNF-alpha acts as a signal to increase apoptosis in a variety of fetal tissues. So the fact that immune stimulation reduces that suggests again that we might be overriding a dysregulating effect on apoptosis by the teratogen.

My student working in diabetes was interested in placenta in part because of the increase in placental weight caused by Interferon-gamma. There are other reasons for this. But evaluated, using an array, he developed in our lab a number of growth factors and cytokines he believed were important in placenta; and evaluated placental function using these.

And very briefly, this line in the urethane-exposed animals represents control level expression of these genes. These are genes expressed at below control level; these at above control level.

With the Interferon-gamma stimulation, you can see the gene expression has increased for the vast majority of these genes he evaluated. With Freund's complete adjuvant, we have more clustering around the control level, more normalization of that gene expression.

So again, he's affecting genes by this immune stimulation-- this, of course, would be predicted--in placenta for genes of this sort. And his theory was that this is related to the reduction in birth defects.

He did a principal component analysis to give a coordinate gene expression picture of this shift. And it was interesting to me how similar this was to our fetal head picture. Here's the control level coordinate gene expression. Urethane caused quite a shift on two axes of this expression. Freund's complete adjuvant normalized that along one axis. Interferon-gamma brought it to beyond normal, and closer on the other axis; beyond normal on one, closer on the other. Here are the immune stimulants alone. All of these treatments affect gene expression.

Is this related to the defect? I don't know, but it was kind of interesting data. It was interesting to me that this profile here was so similar to what we saw in fetal heads of urethane-exposed animals. However, this is a larger panel of genes in placenta.

This student is also a veterinarian, so he's trained in pathology and histopathology; and sectioned placenta and evaluated the effects of the treatments on placental tissue. Here is the syntrophoblast region, the placental labyrinth, this is a control animal, the cytotrophoblast, these are blood vessels.

These aren't the clearest of slides, but I think you can see considerable damage to placental architecture through here in the region of the syntrophoblast. We've got fibrotic lesions through this portion of the slide. That's with urethane exposure.

And now note these lesions. And as we go to the next slide where the animals received an immune stimulation prior to the urethane injection, you'll see that they largely disappear.

And his argument to me was: Think about that. We're improving the support structure for the fetus. If you improve the support structure, then gene expression is going to be more normal. Basically, everything you've seen so far has to do with improving the placenta.

And that sounded maybe a more reasonable argument for the underlying reason this immune protection against birth defects works. It's very believable. But then, immediately you think, "Well, wait a second. Some of these agents--" and again, I can go back to dioxin "--are not placental toxic at levels we're using." There's no placental toxicity of this sort associated with the 9-microgram-per-kilogram dose of dioxin we gave on day nine of gestation. And beyond that, the lesion is well ascribed to a selective effect on cells lining the palate. So while it is attractive for urethane, it's not attractive for dioxin.

I think in the long run we're going to find that it's a multi-factorial mechanism; several different levels are involved. And certainly, improving the placenta would be beneficial to fetal development. And in fact, the fetuses were larger in some cases with these immune stimulations

than in the urethane-exposed animals. So that may be involved.

And that's actually the level we're at in our lab right now. So I am going to stop with that.

[Applause.]

DR. GRUBER: I think we can allow one or two questions. What we're going to be doing is, we're going to change the schedule here. We're going to have the presentation following of Dr. Smialowicz in a moment, and then we're going to have lunch at twelve o'clock. And then after lunch, at one o'clock, we're going to be starting the roundtable discussions.

But you had a question for Dr. Holladay?

PARTICIPANT [In Audience]: Yes. Actually, they are two very brief questions. One, can you please clarify the time sequence in which you gave the immune stimulation with regard to the teratogen? And how much you probed that for how much you could get away with delaying the immune stimulation?

And then secondly, one teratogen which is kind of interesting because it affects immune activation itself is thalidomide, which blocks NF-kappa-B. And I wondered if you looked at that?

DR. HOLLADAY: Those are both very good questions. The immune stimulation timing is important. For instance, with diabetes, if we stimulate after development of

hyperglycemia, we can't block the defect. Stimulation has to occur at a time of normal glycemia.

Now, how early we can go is somewhat surprising, as well. Typically and in the papers in the literature immune stimulation was during gestation. But we found we can immune stimulate these animals actually prior to breeding them, and we still get a significant reduction in birth defects. So that again seems to be somewhat in the phenomenal range.

The whole research area I think is very intriguing. But you can immune stimulate quite early, and still get significant protection against birth defects in a mouse. Now, the second question, which was also a great one but now has slipped my mind--Give me two words. What was that second question?

PARTICIPANT [In Audience]: Thalidomide [inaudible].

DR. HOLLADAY: Thalidomide, okay. Well, we've not used thalidomide. But it raises another interesting issue, in that so many teratogens are also immunotoxic, and I'm an immunotoxicologist. And I hadn't really made this connection before, but all of the teratogens we've worked with here, the chemical teratogens, are also immunotoxic. The dioxin is a wonderful example. And it raises the question, if maternal immune stimulation reduces teratogenesis, how about the flip side of that? Is maternal immune suppression in itself an event that

increases risk of teratogenesis? And thalidomide would fit well into that picture. And I don't know the answer to that. But to me, it's become an interesting question.

DR. GRUBER: I would like to thank Dr. Holladay for his interesting presentation. And I would like to introduce the last speaker before lunch break, and that is Dr. Ralph Smialowicz. He received his Ph.D. from the department of microbiology and immunology at the University of North Carolina at Chapel Hill, School of Medicine.

He is with the U.S. Environmental Protection Agency, at the Research Triangle Park in North Carolina. And his adjunct appointments include the curriculum in toxicology, School of Public Health, at the University of North Carolina, Chapel Hill; and the School of Veterinary Medicine, North Carolina State University in Raleigh, North Carolina.

And I thank him for being here today to discuss further with us the area of developmental immunotoxicology. Thank you.

DEVELOPMENTAL IMMUNOTOXICOLOGY

PRESENTER: RALPH SMIALOWICZ, PH.D.,

U.S. ENVIRONMENTAL PROTECTION AGENCY

DR. SMIALOWICZ: Thank you, Marion.

This is going to be quite a divergence from the discussions and presentations that have occurred thus far. The Environmental Protection Agency is not interested in vaccines. It's interested in environmental chemicals that

humans are exposed to. And consequently, the work that we do deals with that.

What I would like to do is to talk to you about developmental immunotoxicology in a rodent species, primarily in the rat, and some of the work that we have done to demonstrate the efficacy of doing this kind of testing to identify developmental--

[Tape Change.]

2B

DR. SMIALOWICZ: Now, let me get all the equipment together here and start.

I want to congratulate Dr. Insel on his presentation of the development of the immune system. He did it from the standpoint of the human. I'm going to do a quick look at the development of the rodent--this is primarily mouse work--and identify what we consider to be periods during immune system development in the rodent that are critical in regard to when dosing occurs.

If you look at this, you can see that stem cell formation is a critical period. Stem cell formation occurs early, at about the time of circulation, onset of circulation, within the rodent species. The splanchnopleura, the AGM region, gives rise to the potent stem cells that feed to the liver, which in turn seed the thymus and the spleen, the thymus earlier than the spleen. And then eventually, the bone marrow takes over for the production of hematopoietic cells in the rodent.

After birth, we know that in the rodent that the spleen continues to provide B cells to the infant or the neonatal mouse and rat. And we also know, based on the information from many different studies, that this first month of life in the rodent really can be considered a very immunodeficient period of time in the mouse.

As we go through the life of the animal, obviously, there is the establishment of immune memory, which occurs up to six months; and then immunocompetence; and then finally, immunosenescence.

These are some of the markers for B cell development in the rodent species, the presentation of B cell precursors that are found from the AGM period. And this is a time line here. Basically, we get the hematopoietic stem cells getting into the different compartments for hematopoiesis at about day eight.

And then we look at surface markers that Dr. Insel talked about earlier, and the development of the B cells now in the liver.

And then finally, the spleen continues to be the source of hematopoietic stem cells for the mouse. And basically, that occurs after birth with four weeks of life, basically coming to full maturity in the rodent.

This is the hematopoietic scheme for the human. And I'm not going to go through that, since it was covered earlier. I just want to indicate the big difference, as was

indicated earlier by Dr. Insel, about the fact that the rat and mouse, the rodent species, are much less developed at birth than is the human for immune system responses.

This is basically an old slide that demonstrates the contribution of IgG, which Dr. Insel covered earlier, in the fetus, and then the loss of that, and then the production of antibodies by the fetus during the first year of life. So I won't go into that in any detail.

This is T cell functional comparisons between mouse and rat, from Mosier several years ago. This is the mouse at birth, and this is the human at birth. They are responses that are detectable in the mouse at birth, PHA stimulated responses and the mixed leukocyte response, at this early age.

However, ConA and the cytotoxic T-lymphocyte response don't occur until much later in the life of the mouse. However, for both of these types of responses the human is capable of doing that at the time of birth, or earlier.

This is kind of a comparison of several different maturational landmarks, if you would, between the human and the mouse. And this is based on decimal portion of the respective gestational period. We give the human as a 40-week gestation period, and the mouse about 20 days. And what you can see from these different landmarks, maturational landmarks, is that the mouse is much slower in demonstrating these during its gestational period.

There was a question earlier about functional NK cells. And I have a reference to this particular decimal, the activity of natural killer cells in humans that occurs at about a third of the way through gestation of the human. And that was worked by an Italian. I believe it was Santoni [ph]. But if that individual is interested, I could get that reference to them.

So what we have here in the rat and in the mouse is what we would consider the vulnerable periods of immune development, or potentially vulnerable periods of immune development: The hematopoietic portion, which is about day seven through nine; stem cell migration, progenitor cell expansion, day nine through 16; bone marrow and thymus colonization, which occurs from gestation 13 through birth; and then the maturation to immunocompetence, and an establishment of immune memory, from birth to 30 days, and then 30 to 60 days, consecutively.

And what we have done is try to expose animals during this section of the development of the rat, as well as through the entire, or most of, this period of gestation. We haven't done any work during the initiation of hematopoiesis. Basically, all the work that I'll show you, at least from my lab, is from gestation day nine up to about 42 days of age in the rat.

When we do immunotoxicity testing, we have a paradigm that we employ to look at different aspects of immune function.