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DEPARTMENT OF HEALTH AND HUMAN RESOURCES
FOOD AND DRUG ADMINISTRATION
CENTER FOR BIOLOGICS EVALUATION AND RESEARCH

**Public Workshop on Animal Models and Correlates
of Protection for Plague Vaccines**

**FDA's Center for Biologics and Evaluation
National Institute of Allergy
and Infectious Diseases, NIH
Office of Research Development
and Coordination, DHHS
Cosponsors**

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P R O C E E D I N G S**Welcome**

DR. MEYSICK: Good morning. I think we will get started. Welcome to the Animal Models and Correlates of Protection for Plague Vaccines Workshop that is being cosponsored by FDA, NIAID, and HHS.

I am Karen Meysick from FDA. Before I actually ask Jerry Donlon to come up, a couple of logistic things that we need to discuss first.

The workshop is being transcribed, so we ask that everybody use the microphone, and when people come up to ask questions, please identify yourself and the organization you are with. Importantly, the restroom facilities are just straight down the hallway for the gentlemen and straight down the hallway, turn slightly to your right and then on the lefthand side for the women.

Coffee breaks will just be straight out front here in the foyer. Lunch is on your own, but there is a restaurant in the hotel, there is restaurants just in the Marriott, which is about a

five-minute walk away.

The moderator for Session No. 4, who is to be announced, is no longer to be announced, it is Dr. Luther Lindler from the Department of Homeland Security.

There are two replacement sections for your notebooks for Dr. Williamson and Dr. McDonough, just to let you know.

With all that, then, what I would like to do is bring up Dr. Jerry Donlon from the Office of Research, Development, and Coordination at HHS to start us off.

Jerry.

DR. DONLON: Thank you, Karen.

I want to welcome you all to this essential workshop on behalf of the Assistant Secretary for Public Health Emergency Preparedness, the Secretary of my office basically. I also want to thank Drusilla Burns and her CBER team, and the NIAID participants for putting this workshop together.

I think it is a very critical workshop to

advance the development of vaccines for plague. Also, I want to thank the many participants for taking time out of your valuable time from your critical work to attend this workshop and contribute to the discussions, and, hopefully, the consensus at the end of the workshop.

Over the last two or three years, during our experience in looking at developing countermeasures for bioterrorism agents, it became very clear that developing appropriate animal models was a very critical step in the development process, and especially when we come to implement Project Bioshield, which is the acquisition of countermeasures for the stockpile, this process basically is looking at acquiring products for the national stockpile that are still in the developmental phase, but are usable when they are put in the stockpile and eventually licensable.

It is a very somewhat risky process because these products are in the development stage, and it is an accelerated development, and if these products are not, shall we say, placed in an

appropriate development process with the appropriate animal models, we are going to lose valuable time in the acquisition of these products.

So, I think it is very essential, when we are looking at development of any product, that the animal models that are used for that development are basically the ones that will carry it through for a usable product that we can acquire to the stockpile, and then eventually a licensable product. We can't at this point afford to be experimenting, if you will, with various animal models prior to an acquisition.

The confidence in these products that we do acquire for the stockpile will relate to our confidence in the animal models that the results are based on.

No animal model is going to be perfect, and the development of vaccines I think present a specific unique challenge because in addition to asking the question is the pathophysiology of the disease in this animal reflective of the disease in humans, you also have to ask the question is the

immune response in this animal also reflective of the immune response in humans.

So, you have kind of a dual edge task here, one looking at the disease process in the animals, and the other looking at the immune response when you are trying to develop a consensus for an animal model that will reflect vaccines used in a particular disease.

I think that is a unique challenge, and I am sure over the next day and a half, there will be very deep discussions on each of those aspects, the pros and cons. Again, there is no perfect animal model and there will be tradeoffs relative to the pros and cons of the different animal models that will be presented and discussed.

Ultimately, I think it is essential to come to some sort of a consensus, and I think this workshop has both the agenda and the participants to come to this consensus.

It is essential to come to some consensus on a reasonable animal model, not a perfect one, but a reasonable one, to provide guidance and

direction to developers, so that they can apply the appropriate resources and develop the countermeasures in an appropriate time frame without wasting those resources or wasting the time that you are going down a path that are nonproductive.

So, I look forward to the following presentations and discussions as a step forward in developing countermeasures for at least plague. Hopefully, we can develop a consensus and thereby speed the development of these countermeasures for our stockpile acquisitions.

With those opening remarks, I will turn it over to Karen.

DR. MEYSICK: Thanks, Jerry.

The first speaker is actually Mark Abdy from the FDA at CBER, and he is going to introduce everybody into the Animal Rule.

Mark.

Introduction to the "Animal Rule"

Dr. Mark Abdy

DR. ABDY: Good morning, everyone. As I

was sort of chatting with some folks before we got started, I realized that there is many of you that at the very least will know something about the "Animal Rule," and there is many of you that will have attended a talk by someone at the FDA on the "Animal Rule."

My goal today is to go through parts of the "Animal Rule" and illustrate the different questions and concerns that people in CBER will be asking what the requirements will be, so that we can get a plague vaccine licensed using the Rule.

Because of time, I will not address the withdrawal and postmarketing concerns of the "Animal Rule." They are listed in the Federal Register that I will give you the reference for and you can read them on your own if you want to or catch me afterwards.

I hope by this talk I will set the stage for what will be the next day and a half's worth of speakers and discussions. I think I am going to raise issues that will be addressed during these talks and I expect there will be.

Before I get going again, the final thing is I should have some time to answer questions, but again I would ask that you keep them to the generalities of the "Animal Rule," since we will have scientists specializing in plague talking for the next day and a half, and hopefully, your questions will be addressed in the next day and half. Otherwise, catch me in the hallway. I will be here for the next day, as well.

The Rule came about or the idea for the Rule came about in the early 1990s after the Persian Gulf War when the Department of Defense realized that they really didn't have a good mechanism to get the critical drugs and vaccines licensed, and this was for two reasons.

One was the epidemiology of these diseases or agent precludes field trials, which is the usual source of efficacy data, and then the second is that you cannot conduct human challenge or protection studies with certain diseases. It is just not ethical.

So, bringing us back to plague, I think

one of the questions we need to ask ourselves today is which forms of the disease, basically bubonic or pneumonic, will fit the epidemiology issues and the ethical issues.

The official title of the "Animal Rule" is the Approval of Biological Products (New Drugs) When Human Efficacy Studies Are Not Ethical or Feasible.

Before I came to the FDA, I was somewhat naive and I thought that the "Animal Rule" sort of was there as a result of the anthrax attacks in 2001. From the previous slide and this slide, obviously, there was much more going on in the "Animal Rule" in the mid-nineties, and in 1997, the FDA published a Request for Comment in the Federal Register.

It was a Proposed Rule in 1999, and then a Finalized Rule in May of 2002.

You can find the "Animal Rule" in two locations in the Code of Federal Regulations. The first is a new Subpart H in 21 CFR Section 601, and that has to do with biologics, such as vaccines.

The second place that you can find mention of the "Animal Rule" is a new Subpart I in 21 CFR 314, and that has to do with drugs.

To date, only one product has been licensed using the "Animal Rule," and that is pyridostigmine bromide. It was licensed through the Center for Drugs, and all I am going to tell you about it--and I hope I get this right--is that it is a treatment for the nerve agent Somad.

The scope of the Rule is quite broad, it doesn't just handle infectious diseases like we are dealing with today, but it really is drugs and biologicals that reduce or prevent serious or life-threatening conditions caused by exposure to lethal or permanently disabling toxic biological, chemical, radiological, and nuclear substances.

It does not apply if the product approval can be based on standards described elsewhere in the FDA's regulations.

With the "Animal Rule," the FDA may approve a product which has met the human safety standards or the human safety has been established.

That means that you still need to do your Phase I, Phase II, and Phase III studies.

In addition, you have to meet the "Animal Rule" requirements, which will be based on adequate and well-controlled animal studies, the results of which establish that the product, in this case of plague vaccine, is reasonable likely to provide clinical benefit to humans.

One of the misconceptions that we have encountered with some sponsors is that the "Animal Rule" is a shortcut to licensure. I think if you look at what the slide says, you realize that that it is definitely not that, and may, in fact, be a lot more work than your classic vaccines.

But again, we have to ask ourselves, for plague, do we have adequate animal models for plague studies, and hopefully, we will discuss that in the next day and a half.

The Rule is set up on there is four basic requirements for animal studies that have to be met in order for the Rule to move forward, and I am going to go through each of these requirements and

sort of try to relate them to plague.

The first is that there is a reasonably well- understood pathophysiological mechanism of the toxicity of the substance, i.e., plague, and its prevention or substantial reduction by the product, in this case, a vaccine.

Do we have a good understanding of the pathogenesis or pathology of the plague? Do we have a reasonably good understanding of that?

Do we understand how the plague vaccine prevents disease?

The second will be the effect must be independently substantiated in more than one animal species, and this must include species expected to react with a response predictive of humans.

If you read the regs, there is mention of an exception, but as Dr. Donlon just mentioned, I think many people in the audience would agree we don't have, we believe, an ideal plague animal model. We more than likely are looking at two, if not more, but that is up for discussion.

The other thing is we need to know which

animal models, which species and strains are most relevant, and also, does the immune response in these animals resemble that in humans.

The third requirement is that the animal study endpoint is clearly related to the desired benefit in humans, generally the enhancement of survival or the prevention of major morbidity.

In other words, we need an animal model that will show major morbidity or death, because we need to show survival.

So, does the disease, a plague animal model, induce a disease in animals that we see in humans.

The final requirement has to do with kinetics and pharmacodynamics. Basically, these animal studies need to allow for the selection of an effective dose in humans, and to do that, we need to have a good understanding about which components of the immune response are important for protection in plague and how they can be measured.

The second point is we need to be able to bridge the immune response data from animals to

humans.

A brief word on the Good Laboratory Practices and the Animal Welfare Act. The Rule does state that all studies subject to this Rule must be conducted in accordance with pre-existing requirements under GLP regulations and the Animal Welfare Act.

I can tell you that in CBER, we will have the approach that you need to do your animal studies for the definitive or pivotal animal studies according to GLP. You do not necessarily have to do your pilot studies according to GLP, so working out with the correct doses and the correct schedule, it is when you get down to those pivotal studies that they must be done to GLP.

Also, another way that we could think about it is if you want to mention the animal study in your label, then, it should be done according to GLP.

This slide here basically is just a number of bullet points to sort of things to think about when you are designing these animal studies, and

folks in the room that have been working on these are very well aware of these sorts of questions, but you need to think of the label indication - are you looking for a pre-exposure or a post-exposure indication? Are you looking for bubonic and/or pneumonic as an indication on the label?

The route of exposure. We feel pretty strongly that you need to pursue an animal model that will mimic what we expect to see in a human bioterrorism attack. In this case, we are looking at a respiratory model.

Endpoints of animal studies. We are well aware that as you do these animal studies that you have to do your work within the parameters of your IACUC and, in some cases, the European Union regulations, and we will certainly work with that. You do what they tell you to do.

Appropriate challenge dose. This will depend on the challenge route that you choose, the species that you are using, and the strain of *Yersinia* that you are going to use.

Then, of course, statistical

considerations. This is sort of in some ways a no-brainer. Obviously, you can do many more rodents than you can nonhuman primates, and we realize that, as well.

Then, of course, the last point I have here, if you are looking for protection against multiple Yersinia strains, one of the questions that I hope gets discussed in the next day and a half is if we are going to use more than one strain, which strain should be used or tested.

Assays and immunology. Considerable research and development may be necessary to develop and validate these assays. You will need to have validated assays for both animal and human. The human assays will need to be validated before the pivotal or definitive studies.

As far as the immune response goes, I think I have alluded to this already, you must be able to bridge the human and animal data, and then the other thing we would be interested in is the onset of the immune response and the duration of the immune response.

So, to wrap things up, the "Animal Rule" is obviously new to both industry and the FDA, and in order to be a success, we need to collaborate. Certainly, my experience has been that we are doing quite a good job with that on some other agents.

You can expect multiple interactions with FDA Advisory Committees. In some cases--and I don't know what the situation will be for plague--but in some cases, it will be prior to the animal efficacy trials for concurrence with concepts. In other cases, it will be following the Agency's BLA review, prior to approval.

My final slide basically is to recognize that none of this is done by one person obviously. It is a team effort and certainly in the case of IACUC and the plague "Animal Rule," as we move forward with the plague "Animal Rule," certainly Drs. Goldenthal, Burns, Elkins, and Meysick will be very key players.

That is all I have. As I say, if you have general questions, I will try to answer them.

[Applause.]

DR. MEYSICK: What we would like to do now is actually start the session that involves *Yersinia pestis* in general and plague vaccine, so the first session is actually plague pathogenesis. Our moderator for this session is Dr. Susan Straley from the University of Kentucky.

Sue.

Session 1: Pathogenesis of Plague

Moderator: Dr. Susan Straley

DR. STRALEY: Thank you, Karen.

We are going to begin with a general overview of plague pathogenesis that is going to be presented by Bob Perry of the University of Kentucky.

Also, there is going to be a procedural issue that even though everybody can hear the questions that are asked, I am going to need to repeat them up here, so that the transcription will work. That microphone isn't working for the transcription, so we will do that.

Overview of Plague Pathogenesis

Dr. Robert Perry

DR. PERRY: I would like to thank the organizers for inviting me. They actually assigned me three tasks here. One is just a quick overview of the organism, then, to go on to give you an overview of the pathogenic mechanisms or virulence determinants that we know about, and I have chosen to separate these into bubonic and pneumonic plague since they are very different diseases, and the final one was to come up sort of a list of maybe potential new vaccine candidates for subunit vaccine.

Obviously, everyone here knows that *Yersinia pestis* causes bubonic, pneumonic, and septicemic plague. It is a gram-negative bacterium and is a facultative anaerobe, so it can grow both aerobically and anaerobically. I should probably also add it is able at least in vitro to grow in naive macrophages.

The organism is easily grown in vitro. It doesn't have a high degree of nutritional requirements. Genetic modifications are relatively simple to engineer, almost as easy as in *E. coli*.

There are natural foci of infection throughout the world, so the organism can be obtained by going to different locations around the globe. More recently, we have seen some multiple antibiotic-resistant strains that have been isolated from patients, although the degree of development of antibiotic resistance is really extremely low compared to a lot of other bacteria.

Obviously, the organism is infective by respiratory droplet route, and pneumonic plague is very highly and rapidly fatal.

So, all of these characteristics here sort of make this one of the reasons why *Yersinia pestis* is categorized as a Category A select agent.

The other thing that is going on is we currently have no vaccine available, at least in the U.S. and in Europe, and obviously, you are probably also all aware there are several vaccines that are being developed.

So, despite all the concern about potential bioterrorism use, we need to realize that bubonic plague is essentially a zoonotic disease

and it has an obligate flea/rodent/flea transmission and life cycle, so it grows into flea, the flea injects the organism into the mouse or the rodent I should say, and it grows and develops a septicemia, so that now another flea can be infected, and it is this sort of a transmission that you see in nature.

So, I wanted to look at bubonic plague first. I have sort of arbitrarily divided the disease into three stages for convenience of looking at some of the variant determinants we will talk about in a minute.

You can see here that the symptoms, usually from a flea bite, shown right here, usually develop within 2 to 8 days. There is usually a sudden onset of fever, chills, and weakness. Sometimes there is nausea, vomiting, and diarrhea that is also associated with the development of the disease.

Finally, you get a disseminated intravascular coagulation often, and the rate of fatality is between 40 and 60 percent untreated.

If we look at the spread here, it comes from the flea bite, the organism gets into the lymphatics, spreads to a regional lymph node, and you get a large swollen lymph node which has been called a bubo.

From here it breaks out into the blood stream and is spread to internal organs like the liver and spleen where again it grows to quite high populations, and finally, now you have a sustained septicemia, occasional lung infection that can lead to secondary pneumonic plague spread at least in humans, and in 40 to 60 percent of the cases can lead to death.

So, what are the various aspects of the organism that allow it to have this rapid spread and growth in various internal organs and high concentrations of bacteria in the bloodstream, which if you remember, is one of its criteria for being able to survive in nature? It has to develop a high concentration of bacteria in the bloodstream, so a flea can come along and infect another rodent.

Well, there are a number of things that have been studied in the bubonic model, and the first one, and the one most extensively studied, is the type III secretion or low calcium response, and Jim Bliska is going to tell you all about that.

What I just wanted to do here was to show you that this has been extensively studied in all three pathogenic species of *Yersinia*, but in *pestis*, LcrV or V antigen--I always have to have at least one typographical error in all my presentations--the YopH, YopE, and YopM have all been shown to be important in the pathogenesis of bubonic plague. There are some other Yops that Jim will tell you about that really haven't been tested in *Yersinia pestis*. Two of those are YopT and YPKA.

There are iron transport systems, and this is probably what my lab studies, that are important in pathogenesis, and there is *Yersiniabactin* siderophore-dependent iron transport system, and there is another Yfe iron and manganese transport system that play a role.

Finally, Pla protease has been studied for a long time and has been responsible for spread of the organism through different host tissues, and there are some regulators that have been shown to affect the disease course.

One of these is a PhoP/PhoQ, a two component regulatory system. We don't know all of what these regulators control, but they do have effect on pathogenesis. Finally, heat shock serine protease has been tested and also shown to have an effect on virulence.

I have a couple that I have listed under questionable virulence determinants. One of these is the F1 capsule that has been looked at for quite a while. In animal studies, there is really no loss of virulence as at least defined by the crude model of LD50 studies. In some animal models, there is an increase in time to death with this.

I included the Psa, which make fimbria or fibrils. It has also been known as pH6 antigen. In an I.V. model of this, it has a large loss of virulence. In a subcutaneous model, there is

little or no loss of virulence, and this is something we need to look at more closely.

Finally, in the category of things that have been tested, but appear not to have any role in the disease process in at least in bubonic plague models, and these have all been in done in mice, is the Ymt phospholipase D. It has been known as a murine toxin, so you can purify it. Some might purify the protein and kill mice with it very nicely, but it is really not required for the disease process.

By an intravenous model, YopJ really doesn't have a large effect, one of the other Yops that Jim Bliska will be talking about.

My lab has tested a heme transport system and we did not find any loss of virulence again by an LD50 model.

Finally, there is an Hms system that makes a biofilm and that is very important in transmission of plague from fleas to mammals, but the mutation that my lab tested did not find any defect in mammalian disease once it has gotten into

the host.

So, let's go over some of these in a little more detail. I am not going to talk anymore about the type III secretion system, Jim will do that, but what I wanted to do here is start talking about the iron transport systems.

The first one is the Yersiniabactin transport and biosynthesis system. In this model cartoon here, we show that the siderophore, which is a small molecular weight compound that is secreted by the bacterium and has a high affinity for ferric iron, is synthesized by a non-ribosomal peptide synthase enzyme complex, a fairly complex set of enzymes. It is secreted by a mechanism which we have not identified yet.

Once this siderophore or small molecule is in the environment, in our case in the host, we have shown that it is capable of removing iron from lactoferrin and transferrin to the major iron binding proteins that are designed, partially work to keep iron away from invading pathogens.

Once it has bound the iron, it is taken in

through this outer membrane receptor and goes through a transport system to get inside the cell, and the iron is removed by a mechanism which we haven't yet identified. So, if you look at this system from a vaccine standpoint, you have two really targets, the secretory system which we haven't identified and this outer membrane receptor here.

In studies that we have done, if you use a subcutaneous model of bubonic plague in mice, you essentially have a complete loss of virulence. We have no mice die at the highest concentrations we have tested.

If we go much higher with some of the organisms, you will begin to get animals dying of endotoxin shock. However, if you now bypass that first lymphatic stage of the disease by injecting intravenously, these mutants are fully virulent. We have tested mutations in the transport system and mutations in the biosynthetic system, and both of them seem to have equally large effects in the subcutaneous route, but not in the intravenous

route.

The second model is an entirely different type of system. It does not make a high-affinity siderophore defined iron. The system does transport iron. It also transports manganese, and we have a feeling that it may transport zinc, as well, but we don't know for sure yet.

It probably has an outer membrane receptor or a porin of some type through which these substrates channel, but we haven't identified those yet. So, in that aspect, we haven't identified something that is likely to going to be relevant for a vaccine model.

The ions get into the paraplasm where they are bound by a protein and go through the transport system here and get into the cytoplasm. The in vitro growth phenotypes and defects that we see seem to be due to loss of the ability to acquire iron, and not manganese or zinc from our studies, and the animal studies we have done seem to indicate the same thing.

So, if you take and make a mutation in

this Yfe system--and we have generally mutated a, Yba or b, or both, you get about an 84-fold loss of virulence by a subcutaneous route of infection.

Remember I told you the previous iron transport system was fully virulent if you inject it intravenously. Now, if we construct a double mutant system, and this system as well, that mutant is now completely avirulent by an intravenous route of infection.

So, there are a number of inorganic iron transport systems putative and proven in Yersinia pestis genome that at least in the mouse model, it appears that the Yfe system and the Yersiniabactin system are really the only two important ones.

If we go on to look at Pla protease, this seems to be a multifunctional protein. It works to activate plasminogen and inactivates alpha-antiplasmin. It also works to enhance adherence to the extracellular matrix and to laminin. So, one hypothesis is that this activity allows cells to bind to the extracellular matrix and begin degrading it by activating plasmin and enhances

bacterial invasion through the lymphatics.

We also know from studies that have been that it enhances invasion of nonphagocytic cells and again this factor appear to be route dependent as far as its importance goes. So, it is an essential virulence determinant from peripheral routes of infection subcutaneous, has a huge loss of virulence here, over a million-fold, but if you take the same Pla minus mutant and inject it by an intravenous route, it is again fully virulent.

So, the route here, this route dependency seems to sort of support the hypothesis that it may be important in allowing invasion through the lymphatic system.

The two component regulators, PhoP and PhoQ, give you about a 75-fold loss of virulence in a subcutaneous injection model again, and in vitro they survive not quite as well in J774 macrophage-like cell line, about 2.5-fold difference. There has also been a significance increase in sensitivity to growth under high salt conditions, and moderately increase sensitivity to low pH and

hydrogen peroxide.

When the researchers looked at what proteins are expressed, there are a lot of protein changes, but we haven't really identified yet exactly what components this system is regulating. The one thing that we do know that it regulates is a modification of the lipid A structure in lipopolysaccharide, so these mutants lack modification that adds aminoarabinosyl residues.

If we look at the heat shock, which is another regulatory protease, degrades proteins that are no longer functional, again you see a relatively small loss of virulence compared to similar mutations made in other pathogens.

You see also numerous changes in protein expression given that it degrades different proteins, and I should probably have the slower growth at 37 in italics or question mark because the paper that looked at this noted that there was a smaller colony size when you tried to grow the bacterium on a plate at 37 degrees. From this, I would guess that maybe you are getting a slower

growth rate at 37 because of the inability to degrade some proteins.

So, there is a question here as to whether this virulence loss is simply due to slower growth, or whether it is due to loss of degradation of some protein that is normally degraded.

If we get to the F1 capsule, again by a subcutaneous route here--we are looking at bubonic plague right now--there is no change in the LD50. There is a doubling in time to death in a mouse model. There really wasn't a significant increase in time to death in a nonhuman primate model that has been tested.

Despite this, it has been shown that there is an in vitro resistance to phagocytosis that is directly related to expression of the F1 capsule. There is no question that it is a major immunogen and that it is a protective antigen in both bubonic and aerosol models of plague.

Also, the production of this protein and associated components is increased at 37 degrees, so it is going to be highly expressed in vivo.

Now, on the down side here, the mutants obviously in the F1 capsule really don't have a drastic effect on the virulence of the organism, and a little more disturbing is that back in the sixties or so, there were mutants isolated that still make the capsule, but it is no longer cell associated. They are actually secreted into the medium, and what the researchers both in the U.S. and in Russia found is that animals that had been vaccinated with F1 now succumb to the disease much earlier, so it was no longer protective, but it actually helped kill the animals possibly due to anaphylactic shock. These strains have been isolated in both Russia and the U.S. back in the sixties, but not much has been heard of them since. So, this is sort of a word of caution here.

For the pH6 antigen or Psa, it makes fibrils again by an I.V. route in a genetically engineered constructed mutant, you get over a 200-fold loss of virulence. This is bypassing the first lymphatic stage of the disease.

My lab constructed a different type of

mutation, again a large deletion, and we tried this is in a subcutaneous model and really didn't see a whole lot of virulence lost. We think these data are probably pretty good, but it needs to be more thoroughly examined than we have really done to date.

So, it may be a higher degree of virulence lost than would be indicated by the initial studies that we have done here. This system forms fibrils at 37 degrees under acidic conditions. That is why it is called pH6 antigen. It has been shown to be expressed inside of macrophages, and the recombinant Psa protein will actually bind human IgG.

So, to get back to the stages of disease here, to make a point, in that first lymphatic stage we see two processes that seem to be essential or at least very important, and that is the Yersiniabactin iron transport system and the Pla protease. If you have mutations in these systems, the organism is avirulent as long as you have to go from a subcutaneous route.

Once you get to the bloodborne stage here, these two factors are not critical. You don't see a loss of virulence in mutants. What is important now is the Yfe system, we conclude is probably more important in the latter stages of the disease here.

So, that is sort of the stages here, and I want to go on to consider two other systems that are related to growth in macrophages, and the first one is the Hmu heme transport system, and I have already told you that that wasn't important by a subcutaneous route of infection, but it is essential for the use of a variety of heme and heme protein compounds.

You see all these compounds here are utilized by *Yersinia pestis*. If we make a mutation in this outer membrane receptor, which could be a vaccine candidate here, the organism can no longer use any of these compounds as iron sources for growth.

So, in this system, it is likely that the outer membrane receptor binds heme, and the various heme protein complexes, hemopexin-hemoglobin.

Probably the heme moiety is removed at the surface here, taken into the paraplasm, and then transported into the bacterial cell.

There is one protein Hmus that may be involved in removal of iron, so it can be used as an inorganic source of iron, or it may simply bind heme to relieve toxicity of excess heme in the bacterial cytoplasm, and we are not really sure at this point what is going on with this one protein.

So, why am I mentioning this? It is because this system is required for growth in J774 cells. If you look at the graph here, it is actually showing a mutation, a double mutant in the Yersiniabactin and Yfe system, and this essentially acts like wild type. You have an initial death phase and then you have a regrowth of the organism. However, if you have a mutation in the Hmu system here, you have the death phase and they never recover.

So, this is really a system that is required. It's the same if you have only the Hmu mutation and all the other iron transport systems

are effective, you have the same type of curve here. So, this is required for growth in macrophages, at least in vitro, or macrophage-like cells.

The other thing that we found, our Yfe system, which is shown to have some importance in the bubonic model, together with Feo, which is a ferrous iron transport system, which we have a double mutant here, they essentially mimic the lack of growth that you see with an Hmu mutant.

So, these two types of systems, the ferrous iron transport systems and the heme system, seem to be important for growing in macrophages. Whether that is going to be important for the disease process remains to be determined, but either one of these, these seem to be redundant system, and when you take a single system, they grow fine. We need to have deleted both of these for the ferrous iron transport systems.

So, that is basically what we know about bubonic model. Let's go on to primary pneumonic plague.

Symptoms develop in 1 to 3 days after exposure. It develops into a bronchopneumonia, becomes lobar and multilobar in nature. You often have gastrointestinal symptoms like nausea, vomiting, abdominal pain, and diarrhea, and in this case, the disease essentially has a 100 percent fatality rate if untreated, and worse yet, even if you delay treatment more than 24 hours past the onset of symptoms, which are basically flu-like symptoms, then, often it is too late to save the patient.

Now, this model has not been nearly as well studied to date, although that is changing, as the bubonic model, so we don't know as much about the proven or presumed virulence determinants in pneumonic plague.

What has been tested is again the Yersiniabactin mutant although I should have put up here that this is more than just Yersiniabactin mutant. It is a large release in the chromosome, so it is taking out more genes than just that.

There is about a 42-fold loss of virulence

in the mouse model. In the monkey model, LD50 couldn't be figured, but it did alter the disease pathology and the time to death.

Pla has been tested recently, a large loss of virulence as a single mutation, and as a double mutant, here again this is not just loss of Yersiniabactin, but other genes, as well, from a large chromosomal deletion. This mutant was completely avirulent as tested.

The F1 capsule has been tested a number of times. Usually, there is no change in the LD50, there is an increase in time to death in the mouse model, not in the nonhuman primate model.

You will also notice that remember F1 is supposed to be anti-phagocytic and that in the lungs, they did see more bacteria that seemed to be residing in macrophages although it wasn't clear that this was effective in killing the organisms.

So, there are many potential virulence factors determinants that haven't yet been tested. The type III secretion system or low calcium response hasn't been tested at all yet. I think

almost all of us that work with any Yersinia would probably agree it is not going to be as important by this route as they are by the bubonic model route, but the fact is they haven't been tested yet.

The iron transport system Yfe has not been tested and maybe Feo. There is some indication from early literature that maybe there is more of an intracellular phase here in the lungs, so this, and the Hmu heme transport system might have some effect in an aerosol model or pneumonic model of plague, and also, the Psa fibrils pH6 antigen have not really been tested.

So, like I said, there is not as much work has been done on the pneumonic model. That is changing. Let me go over some of the things I hope I have highlighted here as potential new subunit vaccine candidates for a next generation.

The first one is Pla protease, and the pluses here are that it is more highly expressed at 37 degrees, it has roles in adherence/invasion and spread through the body tissues. The negative

aspect is Pla antigen was tested and wasn't found protective. This was done at USAMRIID. I listed it as unpublished, the data wasn't published. It was a line in the paper of another vaccine study.

The Psn, outer membrane receptor for the Yersiniabactin siderophore. Again, the positive for this is it is essential in the early stages of the disease. It is highly expressed in vivo because of the iron-deficient conditions in the host.

The negative here is it is not essential in the later stages. Once you get past the lymphatic stage and into the bloodborne stage, this is not an essential determinant of virulence.

There are a number of outer membrane components, maybe outer membrane components of the Yfe and Feo transporters. Again, they are important, well, Yfe is important in the later stages of the disease. Again, it is going to be expressed because of the iron-deficient environment of the host, and together, these two seem to be important for intracellular growth at least in in

vitro models of macrophage-like cell line.

The negative here is we haven't identified any surface-exposed component to use as a vaccine component.

Ph6 antigen, the fibrillar subunit again is highly expressed at 37 degrees under acidic conditions. Again, we had sort of a contradiction in its role in virulence, and we are not sure what, even if it is involved in virulence, what its role is.

Some studies have shown initially that you don't get a good immune response to just the native protein by itself.

We have the Hmu receptor, again highly expressed as required for growth intracellularly, but there is no role in virulence in the bubonic mouse model, and there are a number of other surface-exposed proteins, secrete proteins, outer membrane receptors, auto-transporters, a number of adhesins and pili that are encoded in the genome.

I point out two recent papers, a signature-tagged mutagenesis, which is going to

identify factors that are important for in vivo growth, and there were a number of things that were identified although not many of them were surface exposed. I think Dr. Titball is going to talk to you about one mutation that was identified that might be the basis of an attenuated lyback seinstrone [ph].

Then, Vladimir Motin and others have done a microarray analysis to look for temperature regulation of proteins, and they found quite a number that are more highly expressed at 37 degrees than at 26 degrees. Now, the caveat here is that we don't know, some of these haven't been shown to be expressed in vivo or to be important in vivo, and so we are at the very preliminary stages of identifying these things.

Finally, there is some cell envelope carbohydrates. F1 is supposed to have a carbohydrate component, but that is not really clear yet, then, maybe the lipo-oligosaccharide--it is called that because it doesn't have an O antigen on it--at 37 degrees it might be investigated.

Although I talked about the problems with F1 protein, so the carbohydrate component may have the same problems, and also with LOS, isolates that were grown at 28 degrees did not provide protection in a bubonic model, but that may have been the wrong temperature, or it may need to be used in combination with other things.

So, with that, I will stop and be glad to take any questions. [Applause.]

DR. STRALEY: Jim.

DR. : Do I need to speak into a microphone?

DR. STRALEY: Speak into a microphone for the audience and then I will repeat it.

DR. : Do you have an idea why the Ybt system is so important in the peripheral route, but not the I.V. route?

DR. STRALEY: The question is why is Ybt so important in the peripheral route, but not the intravenous.

DR. PERRY: We don't have definitive proof. There has been a study that has been done

in *Yersinia enterocolitica* where the systems are essentially identical that shows that the system gets expressed in the liver, in the lungs and the spleen, so it doesn't appear to be a selective expression problem in vitro.

My current hypothesis is, you know, we used to think of the host as, you know, the host environment, and then there is the environment out in the water, but each organ system has different microenvironment conditions, different iron sources, different oxygen and redox potentials, and that might be the case that the system is effective in some organ systems, but not in others, and that is my best guess so far.

DR. STRALEY: Could you identify yourself.

DR. MIZEL: Steve Mizel, Wake Forest University School of Medicine.

My question is, is there any evidence that with the LOS, these organisms can take on phosphoryl choline?

DR. STRALEY: With the LOS, can it take on phosphoryl choline?

DR. MIZEL: In other words, for example, that is thought to be actually a virulence mechanism because of reduced inflammatory responses for the phosphoryl choline associated LOS?

DR. STRALEY: So, does phosphoryl choline reduce potentially in pestis, reduce inflammatory responses?

DR. PERRY: I recently reviewed all of the LPS literature in pestis. I am still not an expert on it, and I can get confused easily, but there is no indication that there is that sort of a modification.

There are other temperature modifications, acidic environment modifications, and some of those do reduce the immune response to the LOS, particularly when you grow at 37 degrees, there is a reduction in the immune response.

DR. STRALEY: While the next questioner is coming, I would like to ask, do we feel that we really understand the modulatory effect of LOS in disease, the effects on the host? For example, as it may relate to toxicity of other factors or as an

adjuvant or literally direct toxicity?

DR. PERRY: So, what is the question again?

DR. STRALEY: We don't talk very much about LOS in pestis and LPS, and yet it could be very important, and I am not sure that we understand its pathogenicity very well.

DR. PERRY: Right. Most of the studies that were done were like in maybe the fifties or sixties, and a couple studies found that it really, compared to other LPS's, is really not very reactive compared to others, at least after they have isolated it.

Now, what its role is, obviously, there are modifications that go on through the PhoP/PhoQ system that tend to help other pathogens survive in an intracellular environment, and these clearly are having research and modification, so you are right, there may be more of a role for LOS in pestis than anybody has been looking at so far.

DR. STRALEY: Olaf.

DR. SCHNEEWIND: [Inaudible.]

DR. STRALEY: While Perry thinks about this, I will introduce Olaf Schneewind from the University of Chicago, and he is asking, do you really need to have something be a virulent factor from all routes, and how would this be measured from the pneumonic route?

DR. PERRY: I have not been an advocate of it has to be essential from all routes. I think we are probably a little better although it may complicate matters quite a bit to have subunit vaccine that has more than two components.

So, you can have things that will be essential by some routes, but not by others. Obviously, the things that are route dependent aren't going to be good, single subunit vaccine candidates, but I view them as may be important in a mixture of components that will help.

Now, I am not really a vaccinologist, I don't know how having five components as opposed to two is really going to complicate matters for the industry folks that are making it and trying to get it approved. It also adds a little bit of

production if you actually believe there is going to be some engineering of these for bioterrorism use to have more components than just a couple.

DR. STRALEY: I would like to raise another question about one of our favorites, which is F1. We think of this as being pretty inert, and in reading the literature, I have the impression that we don't actually know what it does.

I am wondering if you could summarize what people have said about it just for the audience to think about.

DR. PERRY: Well, it is said to be a lipoprotein capsule that has galactolipid associated with it, but it is unclear whether there is a glycosylation side, whether the galactolipid that was found decades ago is really a co-contaminant along with the purification process.

As far as its structure, you get a lot of different theories on that. Some of them have it forming a layer, interlocked layer over the organism that is quite thick, and in that case, it might really occlude or block some other surface

antigens. It is not clear whether that is going to be a big problem or not.

There is the system you and I were talking about yesterday where an old, what was it, 72 or something, where it stopped to form a pore, and really, it is not known what it is doing, form a pore in a phagocyte--

DR. STRALEY: Or modulate a complement--

DR. PERRY: Modulate a complement was another one, so I think that is another area we really don't know how it works. We have been focused on studying how it is as a vaccine candidate and some other aspects of structural access. We really don't know a lot about it actually.

DR. STRALEY: Question?

DR. FROTHINGHAM: Yes, Rich Frothingham, Duke University.

You are one of the few people in your review who I have noticed recently talking about the flea and how far into the skin it goes, and questions like that.

All of your models, all you talk about are subq and I would be interested in your thoughts about where the flea injects. Is there any evidence about factors that might work intradermally versus subcutaneously?

DR. STRALEY: Rich Frothingham, Duke University. The question relates to the flea route and flea bite and where the flea really injects, and is there a difference in the virulence factors' function for intradermal and subcutaneous.

DR. PERRY: Everything I know about the flea I have read, but in the early literature, there seems to be at least an argument back and forth of whether the flea is a subdural or ID injection. Some of them seem to actually have sort of a chewing process and they feed from a pool of blood, so is that an intravenous process.

You are right, there may be differences between subcutaneous and intradermal. We have always done subcutaneous because they are easier. Probably there needs to be some study that needs to use intradermal. I don't think there is probably

going to be a lot of difference between the two would be my guess. I cannot say for sure.

DR. STRALEY: We need to move on. Thanks, Bob.

Our next speaker is Jim Bliska from SUNY Stony Brook, who is going to tell us about Yop effector proteins in disease pathogenesis, and where, in the title, I assume LcrV is included as a Yop.

Jim.

**The Role of Yop Effector Proteins in
Disease Pathogenesis**

Dr. James Bliska

DR. BRISKA: Thank you, Sue. Thank you to the organizers for inviting me. It is a real pleasure to be here today.

I guess my role here is provide an overview of the role of the Yop effector proteins in the type III secretion system in the pathogenesis of plague.

What I am going to try and do is relate the role of the Yop effectors in counteracting

cytokine production and how that relates also to the role of LcrV in the process of delivering the Yops into the host cell and in counteracting cytokine production.

I just want to mention one thing, which is that a lot of the experiments that I am going to talk about, and the models that have been developed, are based on experiments done with the enteropathogenic *Yersinia*, and just as was mentioned by Bob, not as much has been done in this area with *Yersinia pestis*.

Although I think the general processes are conserved, I think it is important to keep in mind that there could be subtle differences between plague pathogenesis and the enteropathogenic *Yersinia* in terms of how the Yops and LcrV function.

Let me just introduce you to the virulence plasmid. It is also known as the Lcr plasmid and in *Yersinia pestis* it is called pCD1, that encodes the type III secretion system, and at 37 degrees, the operons in the plasmid are expressed and it

assembles a type III secretion system, which is modeled here.

The structure consists of a complex basal body-like structure which spans the bacterial envelope and then a rigid needle or structure which extends from the surface of the bacterium.

Now, the substrates that are secreted by the system are synthesized in the bacterial cytoplasm. There are signals in the proteins which allow them to be recognized by the secretion system. There are protein signals in the N-terminus of the protein, as well as signals recognized by chaperone proteins, which direct them to that secretion system.

Some of these secreted substrates also have a signal in the mRNA, as shown by Olaf Schneewind, which is also involved in targeting these proteins to the secretion system, and as I mentioned, the 37 degrees, the system is expressed, the type III secretion systems are assembled, and in response to host cell contact, the Yops and the LcrV protein are secreted.

Also, in vitro, if you chelate calcium ions, the Yops and LcrV are secreted into that bacterial media.

This is a model of how people envision the type III secretions have been working during bacterial host cell contact. This is a thin section end of a macrophage phagocytosing *Yersinia pseudotuberculosis*, and if we could focus in on a region right where the bacterium is in contact with the macrophage in a nascent phagocytic cup, we would envision the following events are happening.

The type III secretion system is assembled in the bacterial envelope. The bacterium also has proteins on its surface which are recognized by receptors on the macrophage, and they can simply enter a pathogenic *Yersinia*, they have the adhesions, invasin, and you add A, which are recognized by integrin receptors, and this mediates phagocytosis of the bacterium.

I guess in the case of plague or *Yersinia pestis*, it is, in my opinion, the most likely proteins that mediate phagocytosis are complement

proteins, such as C3BI, which would be present on the surface, and those would also mediate integrin-mediated phagocytosis.

The substrates, the Yops and LcrV are synthesized in the bacterial cytoplasm, and then upon close contact, the macrophage to the bacterial cell, the type III secretion system is activated, there is HP hydrolysis to drive secretion.

Probably the first proteins to be secreted are Yop B and D and LcrV, because these proteins appear to be required for the translocation process, and there is evidence that Yop B and D actually form a pore in the plasma membrane of the macrophage. Perhaps this pore is connected to the needle, and the Yops and LcrV are then secreted through the system.

The effector Yops, which are shown in green, are delivered into the macrophage cytosol. LcrV is a very interesting protein in this respect, because it is not only required for the translocation process, but it has been detected in the cytoplasm of the host cell, and also it has

been detected in the extracellular milieu of infected cells.

So, I think it is fairly unclear at this point exactly where LcrV is localized during infection, and if it is localized in different environments, what is its role in those different environments.

Once the effector Yops are delivered into the macrophage, they target several key response pathways, and it is pretty well established that in cultured cell infection models, that the two primary targets of the Yops are the phagocytic pathway of the macrophage and also the cytokine response of the macrophage.

The idea that I want to get across today is that in my opinion, I think the ability of the Yops to counteract cytokine production may be more important in disease pathogenesis than the ability to counteract phagocytosis.

So, this just illustrates the ability of the type III secretion system to counteract cytokine production in macrophages. This is an

experiment done with three different strains of *Yersinia pseudotuberculosis*, a wild type strain which under low calcium conditions secretes all of the Yops shown in this STS page gel, a type III secretion system mutant which secretes no Yops in lane 2, and a mutant which is only detected in YopB, is missing a single protein YopB here, but it secretes all of the other proteins including LcrV.

When macrophages are infected with these mutants, and we measure TNF-alpha ELISA, we observe that the wild type strain suppressed TNF release. The two mutants did not suppress TNF release, and, in fact, the YopB mutant was most effective in this response.

So, this told us that the ability of the bacterium during macrophage infection to deliver the effectors through the translocation machinery was critical for the organism to counteract cytokine production.

We went on to show that the YopJ protein in this particular system was very important for counteracting the expression of cytokine mRNA.

I think it is important to consider that this is really just an in vitro system, and we don't really know what Yops are critical for counteracting cytokine production in vivo during infection. I think it is very possible that multiple Yops play a key role in counteracting cytokine production.

To think about this in a very simplified manner, we considered the different response pathways that are activated in the macrophage during Yersinia infection, and obviously, these are the response pathways that the bacterium wants to counteract.

In this very simplified model, we think that there are three major processes associated with the infection that stimulate responses in the macrophage.

The first would be components of the bacterial surface, such as lipopolysaccharide, which will stimulate TLR-4 signaling to produce proinflammatory cytokines.

Another process would be the phagocytic

process itself. I have shown here the invasin protein mediating phagocytosis, but I think in the case of *Yersinia pestis*, complement-mediated phagocytosis would play this role.

This is known to stimulate calcium signaling which can play a role in the ability of the macrophage to, say, generate superoxide response or to fuse lysosomes with the phagosome. It also generates the phagocytic response. It has also been shown to stimulate cytokine production.

Finally, the act of delivering the Yops through the pore induced by YopB and D also can stimulate cytokine production, as we have shown recently.

I think you can see that there are least three major pathways that the infection will stimulate a response in the host cell, and all three of these pathways will potentially generate proinflammatory cytokine responses.

In response to the delivery of the effectors into the macrophage, we envision the next step is the action of the effectors to counteract

these responses. So, as I mentioned, there are 6 known effectors: YopO, which is a serine treating kinase; YopH is a protein tyrosine phosphatase; YopM is a leucine-rich repeat protein. It is the only Yop that doesn't seem to have an enzymatic activity, but it seems to play a role as a scaffolding protein, and as Sue Straley has shown, also localizes to the nucleus of the host cell.

The other 3 Yops are also enzymes. YopT is a protease. YopP, also known as YopJ, is a protease, and YopE is a GTPase-activating protein, which downregulates multiple Ro GTPases.

To sort of categorize the effect of the different Yops on host responses, I am just presenting responses that are targeted by the Yops underneath each name to try and simplify this, and I am using a color-coded scheme to try and illustrate processes that are either unique to a given Yop or that affect cytokine production.

As you can see, there is quite a bit of redundancy in terms of how Yops counteract phagocytosis. So, 4 Yops have been shown to

counteract phagocytosis: YopO, YopH, YopT, and YopE.

On the other hand, some Yops clearly have unique functions, for example, YopH is the only Yop that counteracts calcium signaling. YopM is the only Yop that has been shown to lead to depletion of NK cells in vivo, which has been recently shown by Sue Straley's lab, and YopP is the only Yop that seems to inhibit the survival response of macrophages, which can lead to apoptosis.

Finally, as I mentioned, there is evidence that there are three Yops that can counteract cytokine production: YopH, YopP, and YopU.

When we look at the enteropathogenic *Yersinia*, and we consider which Yops are really important for pathogenesis in a mouse model of infection, it seems like those Yops that have unique functions or that counteract cytokine production seem to be the most important, and those are YopH, YopM, and YopJ, as well as YopU.

This, I think is nicely illustrated in this recent experiment published by Jurgen

Heesemann's group where they tested a panel of defined Yop mutants in a mouse infection assay with *Yersinia enterocolitica*, and they were measuring colonization of the spleen over time after an oral infection.

What they observed was that a YopH and a YopM mutant were the most effective. The bacteria basically never reached the spleen. The YopE mutant and the YopP mutant were partially attenuated in that they reached the spleen, but then were eliminated from the tissues by the immune response.

Then, on the other hand, the YopT mutant and the YopO mutant were essentially as virulent as wild type, so that these Yops, at least in this infection model, are not required for pathogenesis.

So, just to drive the point home again, I think that the Yops that have unique functions, such as YopH and YopM, and those that counteract cytokine production seem to be the most important for pathogenesis in this model.

To now turn to the idea of what is the

protected immune response to Yersinia, I just want to briefly go over the evidence that a T_H1 response is protective. It has been shown by several groups, Bob Brubaker's group and Angl1 Ottenwright's group, that 3 cytokines, interleukin-12, interferon-gamma, and TNF-alpha are protective in the mouse model of infection.

IL-12 is secreted by dendritic cells and macrophages. It drives the differentiation of T cells into T_H1 cells. It will also activate NK cells to secrete interferon-gamma. Interferon-gamma activates macrophages, and TNF-alpha is a pleiotropic cytokine, but one of its major roles is to activate macrophages.

So, this simple model from Janeway's Immunobiology illustrates the role of activated macrophages in eliminating facultative intracellular bacteria in a naive macrophage that is infected with bacteria that reside in vacuoles, that is unable to kill the intracellular bacteria, if it can present antigen to a T_H1 cell, activates the T_H1 cell to secrete large amounts of

interferon-gamma. This activates the macrophage and allows it to eliminate the intracellular organisms.

This is a classic experiment from Brubaker's lab, which he showed that TNF-alpha and interferon-gamma together are protective against *Yersinia pestis*.

So, he was priming mice with either TNF-alpha or interferon-gamma, or different combinations thereof, and then challenging them intravenously with a lethal dose of *Yersinia pestis*. When you use either TNF-alpha alone or interferon-gamma alone, there was little protection. However, when you combined both cytokines, there was complete protection against lethality.

He also measured colonization of the bacteria in the spleen. This was an intravenous challenge model, and the spleen is one of the major sites of bacterial replication in this model, and he observed that in the unprimed mice, the bacteria replicated in the spleen very well, eventually

killing the mice.

On the other hand, the mice primed with the cytokines, both interferon-gamma and TNF-alpha, there was initial replication of the bacteria for a couple of days and then the replication plateaued and eventually the infection was cleared over time.

When he did histopathology, he observed that the wild type strain were the classic necrotic lesion consisting of these necrotic foci with extracellular bacteria and poorly populated with inflammatory cells.

On the other hand, in the primed mice, he observed granuloma formation suggesting that granulomas were controlling the infection and eliminating the bacteria.

In my mind, this creates a paradox that has been present in the Yersinia pathogenesis field for some time, and that is: How can activated macrophages protect if Yersinia are exclusively extracellular pathogens?

I have been thinking about this for a while and I think there are three observations that

are really important in this context. First, is that all three pathogenic *Yersinia* are not fully antiphagocytic at early stages of infection, and this was shown first for *Yersinia pestis* in 1959. So, this is both true in vivo and in vitro that even organisms that are producing Yops will be phagocytosed by macrophages.

The second observation is that at low multiplicities of infection, *Yersinia* do not kill macrophages by apoptosis, and I think that low multiplicities of infection are the conditions that are likely to be encountered at an early stage of the infection process. I think this was first shown actually by John Goguen in 1986.

The last observation is that it is well known, as Bob mentioned, that *Yersinia* can survive and replicate in naive macrophages. This was shown by Cavanaugh in 1959 and by Sue Straley in 1984.

So, recently, we have gone back to look at the role of intracellular replication in *Yersinia* pathogenesis, and this just illustrates a typical example, *Yersinia pestis* replicating in primary

murine macrophages that are naive macrophages, bacteria are labeled with GFP, and you can see that after a 24-hour infection that GFP-positive bacteria are replicating just fine in these macrophages, and it is important to point out that these infections were done under conditions in which the bacteria were producing moderate levels of Yox during the uptake process into the macrophage.

Interestingly, also, we have shown that all three pathogenic Yersinia species can survive and replicate in naive macrophages, so that includes Yersinia pestis, Yersinia pseudotuberculosis, and Yersinia enterocolitica. So, I think all three should be considered facultative intracellular pathogens.

So, the solution to the paradox in my mind is that activated macrophages are protective because they can eliminate the intracellular Yersinia and drive a T_H1 response.

Also, I think that LcrV and Yops function together to counteract production of activated

macrophages, and they do this by eliminating proinflammatory cytokine production in vivo.

So, if this model is correct, you would have to assume that macrophages primed with interferon-gamma would not allow intracellular replication, and that is exactly what this experiment shows. If you prime your macrophages with interferon-gamma, and then infect them with *Yersinia pestis*, there is no intracellular replication.

Also, you would have to say that virulence plasmid would absolutely be required for counteracting cytokine production in vivo, and this has been shown by Bob Brubaker's group, as shown in this experiment, in which he was infecting mice with either a plasmid-cured strain or a wild type strain, and then measuring cytokine production in spleens over different days.

When he infected with the plasmid-cured strain, he saw these rapid spikes in cytokine production that then diminished over time. Production of both interferon-gamma in the open

circles and TFN-alpha in the closed circles.

On the other hand, when he infected with the wild type strain containing the virulence plasmid, haplotype 3 secretion system, there was no early rise in the cytokine levels, and only when the mice started to die was TNF-alpha produced at some detectable level.

Bringing all these observations together, we developed this model, which we used to base our experiments on, and it shows a Yersinia bacterium entering into a generic tissue, such as a lymph node, and under these conditions it starts to produce the Yops at moderate levels in response to the host temperature.

Now, the classic concept of Yersinia pathogenesis is that if it came into contact with macrophages, it would secrete the LcrV, be fully antiphagocytic, and enter into an extracellular phase of growth in these necrotic lesions.

However, we believe that, in fact, at early stages of infection, even though the organism is injecting the LcrV, they are internalized into

the macrophage, into phagosomes.

Howeve, by secreting the LcrV, we believe that they counteract the production of proinflammatory cytokines, such as TNF-alpha and interleukin-12, and this prevents NK cells from being activated to secrete interferon-gamma.

Also, as Sue Straley has shown, YopM causes depletion of NK cell populations in vivo, and this would further prevent the production of interferon-gamma.

As a consequence, the macrophage is not activated, the bacteria can replicate intracellularly, and then escape the macrophage to enter an extracellular phase of growth in these necrotic lesions where it can be at high multiplicities of infection, and under these conditions, it can inhibit phagocytosis by neutrophils and also cause apoptosis in macrophages.

On the other hand, if you infect with a strain lacking the virulence plasmid, so it is unable to secrete the LcrV upon contact with the

macrophage, the organism would be internalized, as well, but under these conditions, the proinflammatory cytokines are produced, NK cell levels are not depleted, lots of interferon-gamma is made, the macrophage becomes activated, it kills the intracellular organism, presents its antigens to T_H1 cells.

This results in more activation of macrophages, and the formation of granulomas, which will eliminate any extracellular bacteria that are present in the tissue.

So, then to finish up, I just want to discuss the role of LcrV in this process. It is obviously a very interesting protein, it's multifunctional. It has been known to be a protective antigen for some time. Sue Straley's lab first showed that it regulates type III secretion system. Bob Brubaker's lab showed that it induces interleukin 10.

Several groups, including Sue's, has shown that it is required for actually translocation of the Yops into the host cell, and then most

recently, Jurgen Heesemann's group provided evidence that it stimulates toll-like receptor 2 in conjunction with CD14 to produce interleukin 10, so it seems to be actually a ligand for TLR2-CD14 receptor complex.

Now, this is the structure of LcrV, which has recently been solved by David Xu's group. It is a dumbbell-shaped molecule with a lower lobe and an upper lobe, and these are linked by the handle, which is a coiled-coiled domain.

Now, as I mentioned, it has been known to be a protective antigen and also antibodies directed against LcrV have been shown to be able to protect mice by passive immunization, and under these conditions, interestingly, Bob Brubaker's group also showed that the mice would produce cytokines when they were passively protected.

So, for example, in this experiment, he infected mice with a wild type strain of *Yersinia pestis* after they had been passively immunized with polyclonal anti-LcrV antibodies, and then he measured interferon-gamma and TNF-alpha in the

spleens of mice, and under these conditions, when LcrV activity was neutralized, there were spikes in cytokine production in the mouse tissues, suggesting simply by neutralizing LcrV activity, you could counteract the bacterium strategy to prevent cytokine production.

Now, some functional regions of LcrV have been characterized. This work has been done in Bob Brubaker's group and Dr. Titball's group by Jim Hill, and also some work has been done in Jurgen Heesemann's group.

This is general structured LcrV. It's a 326 amino acid protein, and two regions have been identified that contain protective epitopes. Region I seems to have minor protective epitopes that corresponds to amino acids 2 through 135, and it corresponds to the upper lobe of the dumbbell in this model which is shaded in yellow.

Interestingly, Heesemann's group has shown that a small peptide, residues 31 through 49, can recapitulate the ability of this protein to stimulate IL-10 production in macrophages.

Interestingly, this peptide corresponds to this small alpha helix on the upper lobe of the dumbbell.

The other region is called Region II. Its residue is 135 to 275, and it seems to contain the major protective epitopes. It primarily corresponds to the lower lobe of the dumbbell here, as well as part of the coiled-coiled domain.

For example, monoclonal antibodies that are directed against Region II epitopes developed in Dr. Titball's lab have been shown to passively protect mice, and also work has been done with this monoclonal antibody to show that it can neutralize the Yop translocation function of LcrV.

So, in my mind, the fact that Region II contains the major protective epitopes and antibodies directed against this region can block the Yop translocation function of LcrV, means that this region is absolutely required for Yop translocation function in LcrV.

So, to put this into our simplified model, we envision that there are two roles for LcrV in

counteracting cytokine production. We call one the long-range mechanism, and the other, the short-range mechanism.

The long-range mechanism would involve secretion of LcrV into the extracellular environment during infection. If it binds to bystander cells that express TLR2, that can lead to IL-10 production.

The short-range mechanism is its required function for Yop translocation where it delivers the effectors, several of which also will directly counteract cytokine production in the target host cell.

Antibodies directed against the different regions of LcrV would neutralize these two functions in different ways, so Region I antibodies would neutralize the long-range mechanism, preventing IL-10 production, and Region II antibodies would neutralize the Yop translocation function of LcrV.

To summarize, what I have provided is evidence that Yops function in concert with LcrV to

target several key immune response pathways in macrophages.

We believe that this set of proteins function to counteract cytokine production to prevent the development of a T_H1 response in activated macrophages, and that antibodies directed to Regions I and Regions II of LcrV will neutralize distinct functions. Region I will neutralize IL-10 inducing activity, and Region II antibodies will neutralize the Yop translocation function of LcrV.

I will stop there and I would be happy to answer any questions.

[Applause.]

DR. NATARO: Jim Nataro, University of Maryland.

[Inaudible.]

DR. STRALEY: Jim Nataro, University of Maryland. So, the question has to do with what is more important, to induce interferon-gamma or antibody, TH-1 versus TH-2 antibody against B.

DR. BLISKA: It is an area that I am not real comfortable addressing, but I would say that

what is important is a vaccine that generates antibodies that effectively neutralize both functions of LcrV, and if I had my choice, I would pick antibodies that neutralize the Yop translocation function of LcrV.

I don't think it really matters what immune response drives the production of those antibodies.

DR. STRALEY: I would like a follow-up. So, do you think that it is important--just from now a vaccine standpoint, we are going to stick this in people--that it is important, that it might be valuable or important to toxoid V in some way?

I mean if it is good enough to do the Yops translocation part, would it be satisfactory to use an internally truncated V, for example, that doesn't do the IL-10 thing? Would that be better than putting the whole V in?

I know that people, who are going to talk about the vaccines, will address the extent to which they are toxic.

DR. BLISKA: That is a good question. I

have looked at this a little bit, and I think what has been shown by Bob Brubaker's group is that if you inject LcrV into mice, and then measure cytokine production, yes, you do get IL-10 produced, but you also get some TNF-alpha and interferon-gamma produced, as well.

So, I don't think injecting purified LcrV, which presumably can induce IL-10 production, is going to dampen the immune response, because I think you also get proinflammatory cytokines produced at the same time.

So, I think the evidence is pretty strong that the full length protein works perfectly well as a vaccine.

DR. MIZEL: Steve Mizel, Wake Forest.

[Inaudible.]

DR. STRALEY: Steve Mizel, Wake Forest.

The issue is what about epithelial cells which are really prominent, and I might add endothelial cells, what about the effects on cytokines by these cells?

DR. BLISKA: It is a good question. We

have done some experiments with epithelial cells, and in that model system, it is clear that multiple Yops are required to counteract cytokine production.

In terms of how *Yersinia pestis* affects the pneumocytes in the lung, I think Sue could address that maybe more directly in that I think she has shown that pneumocytes could play a role in actually harboring the organism. The organism might be able to invade into the pneumocytes using the Pla protease, but I think it is an area that just needs more work.

DR. ZYGHER: Norm Zygher, Centers for Disease Control.

I will extend that question further. What is the role of Yops and LcrV on dendritic cell function and regulation of IL-10 and IL-12 considering that dendritic cells are probably first-line responders in skin, and all the focus so far has been on macrophages.

DR. STRALEY: Norm Zygher, CDC. The issue is effects on dendritic cell cytokine production.

DR. BLISKA: It is a very, very important question. To my knowledge, there has been just a couple papers published on *Yersinia enterocolitica* interaction with dendritic cells, and virtually nothing has been published in terms of *Yersinia pestis* interaction with them or *Yersinia pseudotuberculosis* for that matter. So, it is a complete black box, but I think it is extremely important.

DR. FRIEDLANDER: Art Friedlander, USAMRIID. [Inaudible.]

DR. STRALEY: I will summarize this. The first was a comment from Art Friedlander relating to previous work by Allen Sample and their group, that Pla may have effects on proinflammatory cytokines. Specifically, what effects did you say?

DR. FRIEDLANDER: Degraded.

DR. STRALEY: Directly degraded, for example, interferon-gamma. The other one has to do with interactions with phagocytic cells. So, the issue is once you have the bacteria coded with fraction 1, is the type III secretion system even

relevant. I mean do we need to worry about this, and how does that impact our thinking in relation to vaccine development, because we have to consider possible exposure to a fraction 1 negative, as well as fraction 1 positive.

DR. BLISKA: Yes, I think the observation about Pla in cytokines is important to follow up, and in terms of the capsule, I think during a natural infection with a wild type organism, it probably really is important at late stages of infection, when it is being produced in large quantities, to inhibit phagocytosis, for example.

But the issue is if you make a cath 1 knockout, that strain is still virulent, so in the absence of the cath capsule, in our opinion, the type III secretion system still has the dominant role in counteracting these responses.

DR. FRIEDLANDER: I am just suggesting that it has implications as to where and when temporally it may be affected, but the other point is that one might conceivably deliver what was already encapsulated.

DR. BLISKA: This is one thing that I have thought about, is when you think about how someone is going to grow *Yersinia pestis* before they aerosolize it in some type of attack. It might have huge effects on the outcome, whether the organism is going to grow 27, 28 degrees. If you grow the organism at 37 degrees, you then have to store it for a while before you can aerosolize it, so how is that going to affect the outcome.

DR. STRALEY: This is unpublished data, but we have done some experiments that indicate that antibody against V doesn't have any effect very early on. If you look in the first 6 hours of infection, antibody against V, in terms of colony-forming units viability, it has no effect. It is doing other things, I am sure.

So, I think this is almost moot that NIV is going to protect no matter what state the bugs are in.

DR. FRIEDLANDER: But that has implications about how the anti-V works.

DR. STRALEY: Oh, yes, it does.

DR. SCHNEEWIND: [Inaudible.]

DR. STRALEY: We have a comment with Olaf Schneewind from the University of Chicago playing the role of Bob Brubaker. He wanted to emphasize the immunosuppressive effect of V. Then, have Region I and Region II even separated experimentally.

DR. BLISKA: No, I don't think it has. The only evidence that I am aware of that has been published is this Heesemann publication with the peptide.

DR. STRALEY: I thought that Bob's first studies were actually with a truncate. It was with V that is lacking the first 67 amino acids, so it would lack that immunoregulatory part.

DR. SCHNEEWIND: [Inaudible.]

DR. STRALEY: So, the comment, and this is true, it has not actually been formally proven what the antibodies are inhibiting, whether they are inhibiting the immune modulatory effect of V or the type III secretion aspect, and that is I think a very important question.

Next question.

DR. SRIRANGANATHAN: Nammalwar
Sriranganathan from Virginia Tech. [Inaudible.]

DR. STRALEY: The question is given the importance of T_H1 immune response, have we thought about in vivo expressed antigens as immune targets.

DR. BLISKA: It is a good question. As far as I know, no one has been able to identify something that might be expressed in vivo that functions as a peptide to provide cell-mediated immunity against *Yersinia pestis*.

It is conceivable that LcrV could be processed, and processed and presented by antigen-presenting cells during infection, and that obviously, if it generates a response, it could be protective, but I don't think there is anything known about what candidates you would want to look at.

DR. STRALEY: People have even looked for CD8 epitopes on some of the Yops, like YopH, so potentially, although YopH is not protective because it is sequestered, nonetheless, a presented

epitope might be important.

DR. BLISKA: There has been some work done on YopH. Those were clearly nonphysiological experiments that led to the identification of that epitope. It wasn't generated during a national infection, for example.

So, in terms of what might be generated during a national infection as a protective epitope, I don't know of any.

DR. STRALEY: Last question? John.

DR. GOGUEN: John Goguen, University of Massachusetts Medical School. [Inaudible.]

DR. STRALEY: This was John Goguen from the University of Massachusetts, and he is emphasizing the inadequacy of our database, that most of the work has been done with avirulent models, avirulent strain models or conditionally virulent strains, so we need to take that precaution, and much more work needs to be done on the virulent strain.

DR. BLISKA: I would agree.

DR. STRALEY: That concludes this session.

[Recess.]

**Session 2: Plague Vaccines and Assessment
of Immune Responses**

Moderator: Dr. Conrad Quinn

DR. MEYSICK: The next session is Plague Vaccines and Assessment of Immune Responses. The moderator for this session is Dr. Conrad Quinn of the CDC at Atlanta.

DR. QUINN: Good morning, everyone, and welcome to Session 2.

In this session, we have three speakers. Our third speaker and last speaker of the session is Dr. Sue Welkos from the Bacteriology Division, USAMRIID, Frederick. She will be speaking this morning on assays to establish correlates of protection.

Our second speaker is Dr. Diane Williamson, Senior Scientist at the Defence Science and Technology Laboratory. Dr. Williamson's background is on vaccines with particular emphasis on protective and immune responses to plague and also anthrax, and she will be speaking this morning

on the role and attributes of cell-mediated immunity in conferring protection against plague.

Our first speaker this morning in this session is Dr. Rick Titball, from Microbiology at the Defence Science and Technology Laboratory, Porton Down. Dr. Titball works mainly on the molecular basis of bacterial disease with special emphasis on vaccines and medical countermeasures.

This morning he will be speaking on vaccine design and rationale.

We will start this session with Dr. Titball.

Vaccine Design and Rationale

Dr. Richard Titball

DR. TITBALL: Good morning. It is a pleasure to talk to you this morning mainly about the work we have been carrying out at Porton over the past 10 years or so to develop and improve plague vaccine.

For those of you who are looking at the handouts, I just warn you that I sent my presentation to you in two halves actually, because

it was too big to go through the server here, and it seems to have been recombined in an inappropriate way, so the first half of the presentation is now at the back of that pack, and the last half is at the front.

So, starting off by just talking a little bit about plague. Plague is still a disease, which is of concern worldwide. These are countries that reported plague during the period 1970 to 1995, shown in yellow, and probable foci of disease, shown in red.

There are somewhere around 2 1/2 thousand cases of plague that are reported to WHO each year. So, it is a disease that occurs worldwide albeit in a pretty scattered way. There are sporadic, occasional cases of disease in various parts of the world.

But, of course, the reason that we are here today is to think about *Yersinia pestis* as a biowarfare and bioterrorism agent, and this is actually a cutting from one of the Sunday newspapers in the UK, and it was a cutting that was

taken from a paper printed at the end of the 1990s.

What they did is predicted some of the scenarios we might see worldwide in the 21st century, and one of the scenarios they predicted is that maybe biological warfare agents would be used somewhere in the U.S., and I guess, chillingly, that turned out to be remarkably close to the truth.

So, we are here today to talk about *Yersinia pestis* as a biowarfare agent and how we might protect against that, and, in particular, I guess how we might devise vaccines to protect against disease.

If we think more widely about the populations in which those vaccines might be used and/or tested, obviously, at the moment, we use plague vaccines particularly in research, in laboratory personnel who might be exposed to the bacteria, but there are other populations around the world where potentially we might use these vaccines in the future if they become available, improved vaccines.

In particular, of course, what we are focused on today is the military and civilian populations that might be immunized.

So, what I thought I would start off by doing is thinking a little bit about existing vaccines against plague, what are they, how do they work, what is the evidence that they are effective or ineffective as the case may be, and then move on to talk a little bit about the prospects for improved vaccines against plague.

So, starting off with existing vaccines. There are essentially two types of existing vaccine, a killed whole cell vaccine, which is prepared by either heat or formaldehyde inactivation of whole *Yersinia pestis* cells, and those killed whole cell vaccines are given as multiple dose vaccines over a period of several months, and those vaccines are actually used today to immunize laboratory workers and some other selected at-risk populations in the West.

There are live attenuated vaccines like EV series vaccines, typified by EV76, and those

vaccines have been used mainly in the former Soviet Union and in Madagascar, and they are not licensed in Europe or the USA, and they have not been used to immunize humans in Europe or in the USA.

So, thinking about those two types of vaccines, killed whole cell vaccines, a remarkably long kind of history associated with these vaccines, first devised in 1896, when Haffkine was sent to Bombay to investigate the outbreak of plague in that area, and he devised a killed whole cell vaccine, and remarkably, he actually tested it on himself to prove that it was safe.

So, that was the first killed whole cell vaccine, and there have been a whole kind of sequence of killed whole cell vaccines, which all basically contain the same kind of preparation starting off from the Haffkine vaccine in the late 1800s through to the so-called "Army Vaccine" which was developed by the U.S. Army, and then various commercially available vaccines like the Cutter vaccine and then the Greer vaccine, and currently, the only killed whole cell vaccine which is

available is the vaccines produced by the Commonwealth Serum Laboratories in Australia.

As I mentioned, all of these vaccines basically contain the same preparation. They contain killed *Yersinia pestis* bacteria.

The immunization schedules for these vaccines are slightly different, but basically, they all required a series of immunizations over a period of 6 months. So, in the case of the Greer vaccine, this was the immunization schedule leading to full immunity at the end of 6 months.

In the case of the CSL vaccine, it's initially a two-dose immunization regime followed by 6 monthly boosters, so these are vaccines that need to be given repeatedly to apparently maintain a protective level of immunity.

I guess the real critical issue, the really critical issue is what is the evidence that any of these vaccines work or that they don't work, and the best evidence, aside from animal experimental data, the best evidence that killed whole cells vaccines work comes from the use of

this vaccine during the Vietnam War in U.S. servicemen.

There is quite a compelling set of data that indicates that immunization of U.S. servicemen markedly reduced the incidence of bubonic plague in those individuals. So, in this study, what they did is compared the incidence of bubonic plague in immunized servicemen compared with Vietnamese civilians in and around the same area.

What they showed was the incidence of bubonic plague in the Vietnamese was around 333 cases per million person years. In contrast, the incidence of plague in vaccinated U.S. servicemen was 1 case in 10^6 years, so a remarkable reduction in the incidence of plague.

Now, of course, there might be other reasons that explain that reduced incidence of plague, but for me, the really important issue is that they looked at the incidence of murine typhus, which is spread by the same flea vector, and they showed that the incidence of murine typhus was roughly the same in these two populations.

So, clearly, these people were being exposed, potentially exposed to the bacteria, but they appeared to be protected. So, that is probably the best, that piece of data you will see indicating that killed whole cell vaccines actually do work in human populations.

The other evidence really comes from animal studies, and you can protect various animal species with killed whole cell vaccines against *Yersinia pestis* challenge. One of the tests that was specifically developed to enable the licensing of a killed whole cell vaccine was a so-called mouse protection test, and it is a relatively simple test.

All you do is take sera from immunized animal species whether they be mice or guinea pigs or nonhuman primates or even humans, and passively transfer that sera into mice and then challenge them subcutaneously with 100 MLD of *Yersinia pestis*.

There was a nice little formula that was derived for calculating the so-called Mouse

Protection Index where you look at the percent mortality of that group of mice over 14 days, divide that by the average time to death, and anything that is less than 10 is considered to indicate an acceptable level of protection.

So, the Mouse Protection Index test was used extensively for batch release of various batches of killed whole cell vaccine produced in the U.S. over the past 10 or 20 years or so.

So, there are various bits of evidence that killed whole cell vaccines do work, that they do protect against a subcutaneous challenge with *Yersinia pestis*. Conversely, there is evidence that they don't work very well as pneumonic plague.

Again, there are various pieces of evidence pointing towards that. There are a number of documented cases in the open literature by people who have been immunized with killed whole cell vaccines have contracted and developed pneumonic plague, and there are a number of animal studies.

This is an example of an animal study that

we carried out. Porton mice were challenged either by the injected route or by the inhalation route with 100 MLD or 100,000 MLD of *Yersinia pestis*. These are control animals, so there is no survival of these animals. These are animals that have been immunized with the killed whole cell vaccine, and they are reasonably well protected against an injected challenge, but they are not protected at all against an inhalation challenge.

So, there is good evidence that these vaccines protect against bubonic plague. Equally, there is quite a compelling body of evidence, however, indicating that they don't protect very well against pneumonic plague.

One of the particular concerns with any of these killed whole cell vaccines is their reactogenicity. This is taken from the former Greer vaccine data sheets. So, what it does is list the sort of side effects that people reported either the first or the second dose of the killed whole cell vaccine, and you can see the remarkably high proportion of individuals suffered from some