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

Wednesday, October 13, 2004
8:30 a.m.

Marriott Gaithersburg Washingtonian Center 9751
Washingtonian Boulevard
Gaithersburg, Maryland

C O N T E N T S

Session 4: New Data on Aspects of Plague Vaccine Development

Dr. Luther Lindler, Moderator

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 lifethreatening 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 secretary 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 moity 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, secreter 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 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 operans 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 fined Yop mutants in a mouse infection assay with Yersinia enterocolitica, and ey were measuring colonization of the spleen over time after an oral fection.

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

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

So, just to drive the point home again, I ink that the Yops that have unique functions, such as YopH and YopM, and those at counteract cytokine production seem to be the most important for thogenesis 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 Angll 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. will also activate NK cells to secrete interferon-gamma. Interferon-gamma activates macrophages, and TNF-alpha is a pleotrophic 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 TH1 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 e role of Bob Brubaker. He wanted to emphasize the nunosuppressive effect of V. Then, have Region I and Region II en separated experimentally.

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

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

DR. SCHNEEWIND: [Inaudible.]

DR. STRALEY: So, the comment, and this is true, it has t actually been formally proven what the antibodies are nibiting, whether they are inhibiting the immune modulatory fect of V or the type III secretion aspect, and that is I think a ry important question.

Next question.

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

DR. STRALEY: The question is given the importance of T H1 mune 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 ocessed, and processed and presented by antigen-presenting cells ring 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 ightly different, but basically, they all required a series of munizations over a period of 6 months. So, in the case of the eer vaccine, this was the immunization schedule leading to full munity at the end of 6 months.

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

I guess the real critical issue, the really critical sue is what is the evidence that any of these vaccines work or at they don't work, and the best evidence, aside from animal perimental data, the best evidence that killed whole cells ccines 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 s 1 case in 10 /ears, 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

sort of albeit transient side effect following immunization.

So, these are quite reactogenic vaccines, and in the groups of people who are in the UK with this vaccine, it is not infrequent for people to be sick for a day or two following booster immunizations.

So, that is killed whole cell vaccines. What about live attenuated vaccines? They have never really been used in the West, in the U.S. or in Europe, but they have been used in the former Soviet Union, quite extensively actually, and in some of the French Colonies like Madagascar.

It is quite a high immunizing dose, 6 million CFU, and just to relate the way these vaccines work in comparison to the killed whole cell vaccines, after immunization, what you can demonstrate is sera from immunized animals or individuals, that should work in the Mouse Neutralization test, and the Mouse Protection Index is typically less than 10 after immunization, so it is kind of consistent with this vision, this

picture that a Mouse Protection Index of less than 10 is indicative of protection in that passive transfer model.

There are a number of these EV vaccines that differ very slightly. They all have the same heritage. They are all pigmentation mutants actually, so in contrast to wild type strains of Yersinia pestis, which become pigmented when they are grown on certain agars, like Congo Red agar, these EV series strains are nonpigmented, and it is not fully clarified why they are not pigmented. It is almost certain that they have a number of mutations in the so-called pgm locus, and possibly that affects their ability to acquire iron in the way that Bob Perry talked about this morning.

The precise reasons for attenuation of the EV series vaccines at a molecular level is not known. Very reactogenic. In one study in 1970, in the U.S., in human volunteers, it was reported remarkably that 100 percent of people who were immunized with the ED vaccine developed severe systemic reactions.

Some individuals in Russia, who were immunized with the EV vaccines required hospitalization, and it is quite frequent to have severe local reactions surrounding the site of immunization. So, these vaccines are even more reactogenic than the killed whole cell vaccines.

But they are effective, and they are effective apparently against both subcutaneous and inhalation challenges. So, in this experiment, animals were immunized via the intramuscular route with ED76, and then challenged by the inhalation route. All of the control animals died, but all of the EV-immunized animals are protected.

So, in contrast to the killed whole cell vaccines, these live attenuated vaccines do appear to protect quite well against an inhalation challenge.

So, in summary, killed whole cell vaccines, not very good, don't protect against pneumonic plague. Live vaccines, like the ED series, do protect against pneumonic plague, but they are highly reactogenic, and they have never

really been accepted at least in the West.

So, what about an improved vaccine?

A number of approaches one might use to derive an improved vaccine, one might try and derive a live attenuated mutant to replace the ED76 vaccine, a safe live attenuated mutant, or one might try and identify the important protective components on Yersinia pestis and put those in some sort of subunit or make a DNA vaccine.

Starting off with live attenuated mutants, we spent quite a lot of time pool matching, trying to devise live attenuated mutants of Yersinia pestis, and our initial attempts were not particularly successful.

Although we can derive mutants which are attenuated in the murine model of disease, they are nowhere near attenuated enough, like the PhoP mutant, 75-fold attenuated, that is nowhere near attenuated enough for this kind of mutant to be considered as a live attenuated vaccine.

But more recently, there have been some successes. For example, a group in Israel recently

reported that they had isolated a pcm mutant of Yersinia pestis, which was over 10 million-fold attenuated in the murine model of disease.

The map pcm mutant does look like a possible live attenuated mutant vaccine, so this is a comparison of the way in which the pcm mutant on the ED76 strain perform in the murine model of disease. These are responses developed to F1 antigens, so these are F1 antibody responses induced by the pcm mutant and by the ED76 strain, V-antibody and level of protection.

The pcm mutant performs much, much better than the ED76 strain by any of these criteria that are compared in this graph. So, maybe there is a suggestion that some live attenuated mutants can be devised which have improved performance compared to the ED76 strain, but whether these kind of mutants will ever be acceptable for use in humans, I guess is a subject that is open to debate.

Subunit vaccines, we have looked at a whole range of subunits, and I know other people have, like Sue Straley, and so on, have looked at

various components of the type III system as candidates to go into some subunit vaccine.

To date, the only subunits that have been identified that provide good levels of protection, at least in the murine model of disease, are the F1 antigen and the V antigen. We can actually produce these proteins relatively easily using recombinant DNA technology, so to make the F1 antigen, we just transfer the entire F1 operon into E. coli, and that directs synthesis and export and assembly of F1 antigen on the surface of E. coli in much the same way as it would on the surface of Yersinia pestis, and you can harvest F1 antigen quite easily from the surface of the bacteria.

V antigen can be expressed very easily as a GST-fusion, fusion with a carrier protein like glutathione S-transferase, and in the system we use to generate V antigen, you cleave the V from the carrier using PreScission protease. So, we generate what is very close to an authentic N-terminus of the protein.

These individual subunits work very well

as protective antigens. This is experiments in the mice model of disease, so these are challenge doses increasing from 10

5 up to 109 CFU of Yersinia

pestis. This is actually given by the subcutaneous

route of challenge. These are control mice, so they will die at any of the challenge doses that we have tested.

These are mice that are immunized with F1 antigen, and they are protected against lower challenge doses, partially protected against lower challenge doses, but at these very high challenge doses, we see defeat of protection, similarly with V antigen, defeat of protection at very high challenge doses, but when these two components are formulated together, what we end up with is a vaccine that appears to provide very, very high levels of protection at least against the subcutaneous challenge with fully virulent Yersinia pestis.

Not only does it protect against a subcutaneous challenge, it protects very well against an inhalation challenge. So, again, this

is the murine model of disease, mice challenged by the injected route subcutaneously or by the inhalation route.

These are mice that have been immunized with one of the killed whole cell vaccines just for comparison. These are mice that have been immunized with recombinant F1 and V vaccine that we have devised, and as you can see, we can solidly protect these animals against either subcutaneous or an inhalation challenge.

We can demonstrate that protection against a range of different strains of Yersinia pestis including the F1-negative Java 9 strain.

So, that vaccine has been formulated as a two-dose injectable vaccine, and the current immunization schedule involves giving a dose on day 1 and a dose on day 21, and it is projected that it will involve somewhere around 40 micrograms of F1 and 40 micrograms of V antigen.

I guess one of the important questions, one of the questions that has come up from this morning, is that although people have looked for

additional protective antigens that might protect against plague, what is the evidence that there might be additional protective antigens.

This is an experiment we did very recently with a PYV cured strain of Yersinia pseudotuberculosis. It was also actually a dam mutant of Yersinia pseudotuberculosis, but maybe that is not too significant.

In this study, what we did was immunized mice either orally or intravenously with this PYV cured strain of Yersinia pseudotuberculosis and then challenged them with Yersinia pestis, and remarkably, you can protect pretty well after either oral or intravenous immunization with this mutant, and certainly, to us, that suggests there must be other protective antigens out there, but presumably are co-displayed by Yersinia pseudotuberculosis that are just waiting to be discovered. So, I am sure there are additional protective antigens out there.

What I would like to do finally for the next five minutes or so is just talk about the

prospects for third generation vaccines because maybe the vaccine that we are looking at for the moment, that we are developing at the moment, the F1-V vaccine is just one step towards an ideal vaccine against plague.

One of the requirements may be of a third generation vaccine is it can be given non-invasively, hopefully orally, as a single-dose vaccine, and one of the technologies we have been looking at is to transfer some of these protective antigen genes into Salmonella typhi, and in this experiment, what we did was transferred the gene clustering coding the F1 antigen into Salmonella typhi BRD1116. This is an aroA, aroC, htrA mutant. So, this is the same strain that is currently proposed as live, orally delivered typhoid vaccine.

There are typhoid bacteria expressing F1 antigen on the surface, so they actually make F1 antigen, they express it on the surface, and you can demonstrate expression of F1 antigen in macrophages infected with this recombinant Salmonella typhi, and you can demonstrate the

induction of protective responses in the appropriate model of disease.

This is the intranasal immunization model, so in this experiment, what we did is immunize the mice intranasally with Salmonella typhi expressing F1 antigen on the surface, and those mice are reasonably well protected, around 70 percent protected against subcutaneous challenge with 100 MLD of Yersinia pestis.

So, there is certainly a suggestion that we can devise single-dose, non-invasive, delivered orally or intranasally delivered vaccines.

Naked DNA vaccines are another possibility for future third generation vaccines, and there are some various reports out there actually indicating that naked DNA vaccines, which encode the F1- or V antigens are effective, that they induce protective responses against plague, but the problem is at the moment we need to give multiple doses of those naked DNA vaccines.

Often you need to use them as prime-boost strategies, and to me, it is not overly apparent

what advantages this type of vaccine have over subunit vaccine containing just the proteins you are interested in.

So, in summary, what I hope you have taken away from my presentation this morning, the kind of key messages are that the existing killed whole cell or live attenuated vaccines have significant limitations both with respect to their ability to protect against pneumonic plague, their reactogenicity, and the ease with which they would be or could be licensed in for use in humans.

Improved live attenuated vaccines do appear to be feasible, and there is that demonstration of proof of principle with the pcm mutant that I just talked about, but I guess there is always going to be a question about whether we are going to accept that type of mutant for large-scale immunization of human populations.

There are some suggestions that subunit vaccines, particularly based on the F1- and V antigens at the moment appear to be effective and appear to be safe, but there may well be additional

protective antigens out there waiting to be discovered and waiting to be formulated into an improved third generation, fourth generation plague vaccine.

Finally, there is some evidence indicating that orally or intranasally delivered vaccines against plague might become a realizable prospect as a third generation vaccine in the future.

Finally, just a list of collaborators. Most of the people who have been involved in this work have been located at Porton Down, but we have some very good collaborations with the London School in London, very good collaborations with our Swedish colleagues at the National Defense Research establishment in Sweden, and finally, some links with the University of Umea.

Thank you very much. I would be very happy to answer any questions.

[Applause.]

DR. QUINN: We have got about five minutes for questions.

DR. MIZEL: Steve Mizel, Wake Forest.

Do you have any evidence with your intranasal immunizations of any kind of neurotoxicity as seen with several other vaccines? DR.

TITBALL: We have not seen that with the recombinant Salmonella typhi, but I guess the intranasal immunization model is really just a model for all immunization in humans with Salmonella typhi, so maybe it is not necessarily the most meaningful as to whether you would see any neurological consequences.

We have actually, Di has done quite a lot of work giving purified F1- and V antigens intranasally in various microencapsulated formulations, and we have never seen any adverse side effects which indicate neurotoxicity when given by that route.

DR. NATARO: Jim Nataro, University of Maryland.

Several groups have proposed using attenuated pseudotuberculosis or enterocolitica, which obviously have some real advantages. You mentioned one series of studies. But there is

obviously a very high rate of postinfectious sequelae with those infections, arthritis, and even amyloidosis.

I am not familiar with that in pestis, but do you want to comment on whether that is being looked at, at all, or whether those vaccines are impeded?

DR. TITBALL: You are thinking about in people you could immunize with the live attenuated EV76 strain, because most people who are infected with Yersinia pestis, a reasonable proportion go on to die. I guess of those that recover, I am not aware there is any indication of any kind of arthritic complications of sequelae in those populations.

Similarly, I am not aware of any reported indications in Russian populations that have been immunized, but in some of those populations, it is kind of questionable whether those issues would have been recorded appropriately.

DR. NATARO: But do we assume that enterocolitica and pseudotuberculosis are dead as

far as vaccine candidates, because of the risk of postinfectious sequelae?

DR. TITBALL: I think it depends on which serotype of enterocolitica or pseudo-TB you pick. I don't know. No, I wouldn't say they were dead. I don't know whether anybody else wants to comment.

DR. SMILEY: Steve Smiley from Trudeau Institute.

As a follow-on to that question, so with the plasmid cured pseudotuberculosis, the protection you see there, do you know whether that is antibody mediated, can it be transferred?

DR. TITBALL: I have no idea. Those are all really important experiments that need to be carried out.

DR. : In your studies, you mentioned the challenge with a different type of modality, such as subcutaneous, intranasal, and aerosol. So, from your experience, do you think intranasal could in some way reflect pneumonic model?

DR. TITBALL: That is a very good

question. I think the preference is always to carry out an inhalation challenge, you know, if you have that capability, and we would not substitute an inhalation challenge or vice versa, but there is some evidence that actually, intranasal challenge does result in a disease which is very similar to that, that you see after inhalational challenge.

I guess there hasn't been enough detailed histopathology carried out to actually compare the disease in detail after challenge by those two different routes.

DR. : Because that would be very important to actually, intranasal would be much better controlled than the inhalation.

DR. TITBALL: Maybe, but I mean cytodeposition is always going to be slightly different. It depends on whether you are actually talking the deep lung or the upper respiratory tract.

DR. : Right, but actually, most of the respiratory is in upper respiratory infection.

DR. TITBALL: Right, but probably not after exposure to Yersinia pestis used a biowarfare agent. It is much more likely to be a lower respiratory tract which is targeted.

DR. QUINN: We have time for one last question.

DR. : The question I have concerns topics that came up earlier. This was a beautiful synthesis of what you can get with a vaccine.

Richard, what I want to know is did you try that actually at Porton Down on laboratory workers, that vaccine, and what do you think about their concerns about F1 that were raised here earlier and the immunosuppressive role of LcrV, and how do you feel about it?

DR. TITBALL: Those are good questions. I think Di is going to talk about some of the clinical trials we have carried out with this vaccine. Our F1 and V vaccine has been into people, and there are no obvious indications of adverse side effects.

Clearly, one of the reasons for including F1 and V in any future vaccine has to do with this issue of possible virulent strains that lack F1 antigen on the surface, and I guess the issue about the possible immunosuppressive properties of V, you know, it is probably best, though, by considering in those experiments where immunomodulatory properties have been reported, the V antigen has been given repeatedly at daily intervals in quite large doses.

It has not, to my knowledge, been given as a single low-dose cell-purified protein.

DR. : Let me just ask you, what do you think of the 2 "Animal Rule" in terms of plate testing that is proposed to the U.S., because I think you probably work in different coordinates than EU.

DR. TITBALL: Not necessarily actually. I mean it would be the same kind of considerations for us in the UK that we would need to demonstrate efficacy in at least two animal species.

DR. QUINN: We will introduce our next

speaker, Dr. Diane Williamson from Defence Science and Technology Laboratory, Porton Down, who will be talking to us about the role of antibodies and cell-mediated immunity in protection.

The Role of Antibodies and Cell-Mediated Immunity in Conferring Protection Against Plague

Dr. Diane Williamson

DR. WILLIAMSON: Thank you. Good morning and I would like to start by thanking the organizers for inviting me to participate in this workshop. It is a great privilege to be here.

I just want to point out that my hardcopy of my presentation is actually on your supplements in your binders. I reordered the size in order to try and address some of the questions that I thought the panel might be concerned with tomorrow and also to try and prevent duplication of some of the subsequent speakers talking about small animal models, so if you follow my presentation, if you would like to follow the supplement rather than the bound-in copy.

I am going to try to cover the role of

antibodies and cell-mediated immunity in conferring protection against plague. Of course, you have heard that plague is predominantly an extracellular infection with intracellular phases.

So, what does this mean in terms of the immune response? Protection against plague will depend on countering the bacterium and its virulence factors. We have heard already a lot about the virulence factors that this organism is able to produce.

The host, in order to protect itself, will need to induce an appropriate immune response or will need to be induced to produce an appropriate immune response by vaccination, and we are going to talk about antibody and cell-mediated immunity in that context.

Of course, because this is a serious human pathogen, and because field trials showing efficacy are not going to be easily achieved, we need to depend very heavily on animal models to elucidate these protective immune responses.

I want to talk this morning a little bit

about the data that we have gathered so far in mouse, guinea pig, beginning to gather in the marmoset with the small nonhuman primate model, and in macaque, and also some immunogenicity data that we have gathered so far in a safety trial of vaccine in man.

So, just starting with the mouse, what we have here is a very early study where we showed that F1 and V in combination were protective in the mouse model, and this is a BALB/c inbred mouse model, against a human fatal isolate of plague, and they conferred the same level of protection as to live attenuated ED76 vaccine, and, by comparison, the killed whole cell vaccine was defeated against this very high, 10^9 subcutaneous challenge.

So, antibody is probably very important in protection. What I want to do now is just characterize what we know about the kinetics of antibody production in our animal models, quantity of antibody produced. I want to look at the similarities between the animal models, and then try to relate the antibody characteristics that we

have observed to protective efficacy, and then look at the rationale for extrapolation from the animal models to man.

Just looking at the kinetics first, we have looked previously at the antibody response to the F1 plus V combined vaccine in four different haplotypes of mice, and you can see that from this kinetic study where mice were immunized at day naught and at day 21, that antibody response started to rise very fast and peaked at about a week after the second dose of vaccine. These animals were then boosted later on, and cell-mediated antibody was followed right out for several months.

But the take-home message from this slide is that although we have four different haplotypes

mouse here, they are all responding in a very similar way with

eir antibody kinetics.

When we challenged these mice at day 80, we saw some

otle differences in protection against subcut challenge. This is

very high challenge level. It is a very virulent strain of

plague, however, and I don't think these really are very significant differences.

When we challenged the mice by the aerosol route, we saw solid protection at this time point.

So, in these inbred strains of mice, haplotype doesn't seem to have very great an influence on levels of protection achievable.

We also looked at gender within these haplotypes and compared male and female mice responses, and saw little difference there either.

We went on to select the BALB/c mouse for most of our other subsequent studies and here I am showing you a dose response curve in the BALB/c mouse where we immunized with decreasing concentrations of the F1 and V subunits and challenged the mice with 10 7 CFU subcutaneously, or 10 5, and you can see that the minimum protective dose against the 10 5 CFU is around the 1 microgram mark, and the minimum protective dose against the 10 7 CFU is around the 5 microgram mark of vaccine.

We were able to correlate the predominant IgG subcuts or haplotype IgG1 with protection in

the mouse, and showed that as you decreased the dose, you lost IgG1, and that relationship correlated significantly.

We also looked at the protective efficacy of vaccine in outbred mouse strain, and we, at Porton, have an outbred closed colony, outbred mouse strain, which we call the Porton mouse. It is a very stable strain. We immunized these mice with the F1 plus V combination and challenged them at day 60 of the two immunizing doses with plague by the aerosol route, and we showed very solid protection against 100 LD50 of plague by the aerosol route.

We also actually escalated the immunizing dose up to 75 micrograms and gave it on a single occasion in this last part here, and challenged these mice by the aerosol route and showed that we could protect them against 10 4 LD50, which is the maximum protection we have shown against the challenge in the mouse model to date.

Having escalated the vaccine dose up to 75 micrograms, we did some more exploratory work where

we looked at the increasing doses of the vaccine
ven very soon prior to challenge, and here, we have some data which just
ows, in the BALB/c mouse model against an aerosol challenge of 300 lethal
ses, but even giving the vaccine three days prior to challenge, one can get
ne protective effect, and giving it six days at 25 micrograms of each subunit,
e can get full protection.

So, this is quite encouraging data, may translate to the use of
ccine postexposure if you need six days to achieve protective immunity, that
ght set your time frame for postexposure therapy.

Moving on now to the guinea pig. We have
ne some limited work in the guinea pig. The guinea pig, we do not find to be
very good model of plague infection. The plague infection seems to be very
ronic in the guinea pig, unlike the mouse where you have an acute infection,
e guinea pig seems there is a very chronic infection.

When we looked at antibody responses in our guinea pigs, in our
munized guinea pigs, at

time of challenge, we saw very variable responses to the F1 antigen, and much more consistent responses to the V antigen.

When we challenged these immunized animals, we managed to achieve full protection against an injected challenge of 10 5 lethal doses, 10 5 CFU, and then partial protection /ond that.

But we are not planning to pursue the guinea pig too much further as a model because of the difficulties, and you can see that in survivors here, we had very protracted time to death as the infection became very chronic.

We have done some work in cynomolgus macaques, and I am just going to describe to you an immunogenicity study where we looked at ascending dose levels of vaccine in this range in male and female cynomolgus macaques immunized on two occasions.

Here, we have typical antibody response. This is to the V antigen in these animals. I have shown just the 10 microgram, the response to the 10-microgram dose group and the 40-microgram dose

group just for comparison, and you can see that animals were immunized at week naught and at week 3, we get some very nice secondary antibodies form to the booster dose in the green and red bar.

The yellow line here represents animals that were given a single immunizing dose at the 40-microgram dose level of the vaccine, and you can see that they responded reasonably well, but, of course, didn't develop the secondary immune response.

Moving on now to observations of antibody responses in man, we have done a preliminary Phase I safety study in Europe in 32 individuals given the vaccine in the same dose range as used in the macaque study, alhydrogel adjuvant, and we have looked at safety and found absolutely no safety concerns with this vaccine.

We looked at some cytokine readouts, for instance, IL-6, and saw no change in vaccinees in that IL-6 level, and additionally, we were able to do some immunogenicity work with serum from the volunteers, and what we found was that when we

immunized in this dose range, we got this kind of pattern of antibody response. All individuals responded to either of the antigens. Some did not respond to F1, some did not respond to V, but generally, at the 40 microgram dose level, we had complete response to both antigens.

You can see a dose response effect here with increasing agglutinine [?] titers with dose level.

Just turning now to antibody functionality, what I have talked about so far really are observations on kinetics and quantity of antibody, but what does that mean in terms of antibody functionality?

We can look at neutralizing antibody by competitive ELISA, we can actually look at the inhibition of the cytotoxic effect of V antigen as expressed in pseudotuberculosis construct in vitro, and we can look at passive transfer, and I just want to quickly run through the data we have to date in this context.

We have developed, at the research level,

a competitive ELISA for V antigen, and this ELISA depends on coating with V antigen, and then introducing the monoclonal antibody 7.3, which we have previously shown to be protective against plague challenge when given by passive transfer in the mouse.

That monoclonal antibody binds to the antigen and we start with 100 percent binding of that, and then we introduced vaccinee serum at various dilutions. When you introduce macaque serum in this case, 1 in 80 dilution, you begin to see competition with the mouse monoclonal antibodies binding to V and some loss of mouse antibody signal here.

As you increase the concentration of your vaccinee serum, you can see that you get complete inhibition of binding of the mouse antibody to V. Now, we have done that for macaque serum, and we have also used the same ELISA to evaluate our antibody responses in people receiving the vaccine at the highest dose level tested.

What we found was that all individuals at

this 40 microgram dose level had neutralizing antibody for the V antigen and the serum, and also that neutralizing antibody correlated with total IgG, significant correlation with total IgG that those individuals were producing.

Just moving on to in vitro cytotoxicity, there is an assay that we and others are using where you can express V antigen from pestis, from pseudotuberculosis, and that construct is [inaudible] for macrophages in vitro.

You can therefore use this assay to look for inhibition of the cytotoxic effect with your vaccinee serum, and at the moment, this assay in our hands is a qualitative assay. Here, we have some readouts from the assay.

Here, we have macrophage cells in culture which are uninfected and green cells glow green, live cells glow green, and dead cells glow red.

So, you can see that they are predominantly live here.

When you introduce the pseudotuberculosis V expressing strain, together with the protective

monoclonal antibody, you get protection against the effects of V, and you get a predominantly live culture.

Here, we have, though, a culture where we have introduced macaque serum taken on day 1 of a macaque immunization protocol, so you wouldn't expect antibody to the antigen in this serum, and indeed we get almost full killing of the culture.

When you take serum from that same macaque at week 10 schedule, you can see that it now has developed neutralizing antibodies to V antigen, and protecting the culture from killing in this assay.

Similarly, when we took serum from macaques that had been immunized, and we took serum at week 6 or week 10, we got similar protective effects.

So, this assay is giving us qualitative positive readout and showing that there are neutralizing antibodies in sera from these animals.

Just turning now to passive transfer, Dr. Titball mentioned passive transfer as a means of evaluating the vaccine in his presentation. We

have done a lot of passive transfer from the species into the mouse at Porton, and what we have found is that when BALB/c mice are immunized with the vaccine, and this is on 3 occasions, and then the serum is taken and transferred into SCID/Bge mice, and these are severe combined immunodeficient mice with the beige mutation, they have no functional immune system.

We can protect the recipient mice against challenge by the subcutaneous route and by the aerosol route. You will note that there is some breakthrough at the end of this 10-day assay for both challenge models, but this is probably attributed to the half-life of the passively delivered serum decaying and one then gets breakthrough. We now cap this assay or limit this assay to a 10-day assay.

We have done a similar kind of exercise with IgG purified from immunized guinea pig serum, and here we used IgG at two dose levels purified from the guinea pig serum, and got very similar data.

This IgG has been passively transferred into mice, and the mice have been challenged by the subcutaneous route, and guinea pigs given the F1 and V vaccine, their IgG fully protect mice. We were able to fully protect mice with IgG taken from guinea pigs given the existing plague vaccine, which we have supplemented with V antigen to immunize guinea pigs with.

Similarly, we have transferred IgG purified from immune macaque serum into mice and shown that it can fully protect groups of mice.

Now, what we have here are IgG at the 100-microgram dose level of IgG taken from macaques and mice at the different dose level of the vaccine that I showed you before, 5, 10, 20, and 40, and the single dose 40, and you can that IgG taken from those groups at all the dose levels of the vaccine was protected in the 10-day assay in the mice. So, we are able to transfer protective immunity with antibody.

Finally, in the human model, we have taken serum from donors in the 40-microgram dose level

group, human donors, and transferred their serum into mice and shown either full or partial protection of the mice, and we were able to correlate the protective immunity transferred with the IgG content to the donor serum, and there is a significant correlation there.

So, passive transfer would seem to be a useful method of evaluating serological protective immunity, but what we have found actually is that these assays are very relevant very early in the schedule, up to day 28 or so of the immunization schedule, and people responding with maximum serological antibody, but beyond that, some of the correlations start to fall away.

So, what we need to look at also is the cell-mediated immune response, and this is a rather harder function to assess. We have done some T-cell recall responses in BALB/c mice at 8 months post their original immunization, and shown that they do have significant recall responses, particularly for the V antigen.

So, what else can we do, what else do we

do to look at cell-mediated immunity? Cell-mediated immunity is undoubtedly an influence. The IgG subclass profile that we are seeing in these F1 plus V vaccinated individuals from all species indicates that what we are inducing is predominantly a Th2 response, and that is not a surprise, because we adjuvanted our vaccine with alhydrogel, and flow cytometry analysis in species that we have looked at, mouse, macaque, and man does indicate that what we have here is a CD4-positive memory response, which could be either Th2 or Th1.

But we also have some evidence from mouse models that a Th1 response to challenge is also essential to clear the infection, so although our vaccine is inducing predominantly Th2 response, the vaccinees are able to mount a Th1 response, they are able to mount a Th1 response, and that is essential to clear the infection.

Just to summarize very quickly a lot of work that we have done in genetic knockout models, we have looked in genetic mouse models which have a

targeted gene deletion in the STAT 6 pathway, and these animals are not able to mount a full Th2 response, but they do have an intact Th1 response.

Conversely, we have looked at targeted gene deletions in the STAT 4 pathway where these animals in a C27 background cannot mount a Th1 response, but do have a full Th2 response.

What we found with these animals was that a reduced vaccine efficacy occurred in STAT 4 knockout mice, and this correlates with absence of CD4 Th1 response, so that when we immunized these mice in the usual way, and challenged them at day 60 with plague, by the subcutaneous route, we saw breakthrough first in the STAT 4 knockout mice, and STAT breakthrough, but as we increased that challenge dose, we saw full breakthrough.

So, these animals are able to produce, mount a Th2 antibody, full antibody response, but cannot mount a Th1 response, and they are susceptible.

So, it looks as if both Th2 and Th1 responses are required for full protection against

plague, and when we looked a little bit further into this, we collected splenocytes from cohorts of these different strains that we have used in this experiment and re-presented them in vitro with the F1 and V antigens, and showed that whether they have been vaccinated, in the blue bar, or not, in the red bar, these strains were able to produce interferon-gamma in response to resubmission with the F1 and V antigens in vitro, but the STAT 4 models were not, as we expected, and it would seem, therefore, that the deficiency in protection in the STAT 4 mice can be related to lack of a Th1 response.

Just very finally, we are doing a lot of work at the moment looking at trying to map T cell epitopes in both F1 and V antigens, and we have nearly complete maps of the murine T cell epitopes in the V and the F1 antigens.

Now, what we hope next to do is to start to ascribe some function to those epitopes and then maybe to use peptides that represent those epitopes for which we have ascribed function as better

targets for assessing cell-mediated immunity in man as we proceed into our clinical trials.

So, therefore, in summary, we have shown an antibody response in all species that we have looked at with the F1 and V antigen, and this appears to be fairly conserved as we present these antigens in the alhydrogel formulation across species. Functionality is quantifiable in the tests that I have described, for instance, competitive ELISA, the inhibition of cytotoxicity, and in passive transfer.

That is certainly a mixed Th2/Th1 response is required to clear infection, and it would appear that presenting the F1 and V antigens in alhydrogel will induce cross-prime to both those responses. Cell-mediated immunity is the better black box at the moment. We know it is quantifiable by in vitro proliferation type assays. Perhaps by defining the T cell epitopes further, we will be able to provide improved targets to assess cell-mediated immunity more effectively.

Then, finally, there have been a number of

people, very many people involved in this project over the years at Porton. I have tried to list them all. We also have very good work ongoing currently, headed by our project office at Porton, in transitioning the vaccine from research into development, and that this is staffed by people with regulatory and clinical experience.

Of course, we are also indebted to Avecia Biotechnology, who in recent years have been manufacturing the vaccine for us, and we have a very good relationship with Newcastle University.

Thank you. [Applause.]

DR. QUINN: Thank you, Di.

We have time for some questions.

DR. FROTHINGHAM: Rich Frothingham, Duke University.

That was a very exciting lecture and I am delighted to hear how quickly this work has moved along with this combined recombinant subunit vaccine.

You mentioned that T cell epitopes have now been mapped for F1 and V. Is that information

available?

DR. WILLIAMSON: Not yet. We are about to submit some of that data, but it is just being completed at the moment.

DR. STRALEY: Sue Straley, University of Kentucky.

I am curious, in relation to, say, the development of monoclonal cocktails, whether anyone has looked at a difference in efficacy of different isotypes, IgG2A versus IgG1.

DR. WILLIAMSON: Well, we did actually attempt to do that some years ago, but working in the mouse, isolating these isotypes from the mouse in quantity was not easy. We actually attempted that experiment, but really were not able to proceed because we didn't have enough of the polyclonal-derived isotapes.

Strangely enough, many of the monoclonals that we have are IgG1 and difficult, but I am very keen to find any monoclonals out there, IgG2A or 2B biased, that would be of great interest.

DR. STRALEY: So, your 7.3 is an IgG1.

DR. WILLIAMSON: Yes.

DR. FERRIERI: Pat Ferrieri, University of Minnesota Medical School.

Is there consistency among different laboratories in the aerosol challenge, and specifically, my question is, are you pumping bacteria into a chamber, or on the other hand, are you dripping it into the nose and having them inhale it?

DR. WILLIAMSON: Right. We have had extensive interaction with USAMRIID in establishing the aerosol model. We actually aerosolized with Henderson apparatus or Collison [ph] spray, and we conditioned the aerosol appropriately in terms of humidity and temperature, so we got live bacteria deposited into the deep lung in our mouse model. The animals are conscious when we do this, so we have a lot of experience of aerosolizing, and think we can keep the organisms viable.

DR. SCHNEEWIND: Olaf Schneewind, University of Chicago.

The query that I have has to do with the

publication of Jurgen Heesemann, who used isolated macrophages and showed that the LcrV stimulates an IL-10 release. Is that an assay that you feel should be included in studying the antibody response against LcrV, and, if so, would that be useful for mirroring [?] human macrophages?

DR. WILLIAMSON: We actually have some work ongoing with Heesemann's group. I have supplied him with the antigen to look at exactly that. Yes, certainly, we are to see what comes out of that collaboration.

DR. SCHNEEWIND: In this regard, I was interested in the human studies that you are doing, and you said that you had looked at a cytokine response for IL-6.

DR. WILLIAMSON: Yes.

DR. SCHNEEWIND: What time after infection do you study this?

DR. WILLIAMSON: We looked, not infection after immunization. We looked at the recall points for the volunteers two days after immunization regularly and saw no change in IL-6.

DR. SCHNEEWIND: And the studies in animal suggest that these changes occur within the first 24 hours for IL-10 and IL-6.

DR. WILLIAMSON: Yes. This is probably the logistics of running a clinical trial, one can't have volunteers coming back every day, but really I suppose in terms of immunosuppression, we are interested in whether there might be a long-term immunosuppressive effect of vaccine, so that is why we chose those time points. We couldn't see anything.

DR. QUINN: Last question.

DR. MORRIS: Stephen Morris.

I was wondering, USAMRIID has also used the African Green monkey as a challenge model. Could you comment on the considerations that went into your decision to use the cynomolgus macaque as opposed to that particular animal?

DR. WILLIAMSON: I guess we wanted to select a nonhuman primate model. We have available to us the marmoset. We are doing a little bit of work in the marmoset, the small nonhuman primate

model, but that is slightly behind what we have done in the cynomolgus macaque, and really, it was in terms of the previous literature and the availability to us as cynomolgus macaque.

DR. QUINN: Thanks again, Diane.

[Applause.]

Our final speaker in this session Dr. Sue Welkos, Senior Scientist, Bacteriology Division, USAMRIID, Fort Detrick. Sue will be presenting on assays to establish correlates of protection.

Assays That Can Be Used To Establish Correlates of Protection

Dr. Susan Welkos

DR. WELKOS: We have been interested at USAMRIID in developing in vitro assays which might be predictive of immunity to plague in immunized individuals, and most of the focus of these developments has been utilizing the F1 capsule antigen and the V antigen.

The reason behind these decisions, of course, is quite clear by now, and I won't spend any time on it, but many early studies in animals

indicated that both of these antigens are highly immunogenic and highly protective.

Just, for instance, any combination of vaccines, we tried in a model, murine model, immunized subcutaneously and then challenged with Y. pestis strains CO92 compared to the old Greer vaccine, subunit vaccines containing either V11F1 or even better EF1 fusion construct that was made at USAMRIID. All of these provide significant protection and elicited high titers of circulating antibodies.

So, the question then became, can an immunological response to these two antigens be developed, such that it can be developed into an in vitro assay, which would then predict immunity.

Most of the talk today focused on assays we have been working on that mainly deal with the V antigen and the response to V, however, I wanted to spend a few minutes on a recently developed competitive inhibition ELISA based on anti-F1 responses that has been fairly successful and fairly well developed.

Many people contributed to the development of this assay including Drs. Evanovich, Tran Chanh, Dr. Andrews, Dr. George Anderson. In any event, you have heard this before, the basic outline of the competitive assay utilizes plates that are coated with the antigen, F1 here, and then dilutions of standard known anti-F1 monoclonal prepared, standard inhibition binding curve, and then unknown serum samples are similarly diluted, to each is added a competing antibody labeled biotinylated anti-F1 monoclonal antibody in this case.

Then, the plate is incubated and developed with a rabid anti-mouse stripped out of it, and conjugate. The bottom line of this assay is that in tests done with serum from mice that have been immunized with F1, there has been a very good correlation between the levels of competing F1 antibodies in this situation and protective immunity.

This just gives a summary of one study done with 163 mice that were immunized and then

challenged subcutaneously. It is plotted such that there were several different dose groups of animals that received the vaccine ranging from 0.1 to more than 10 micrograms.

This gives their level of competitive ELISA anti-F1 antibody. You can see that the nonsurvivors are shown in pink, purple and pink, those individual quantities circulating F1 specific antibody, and in blue are the survivors, and if the means of these two groups are calculated--it is not shown here--but the mean of the nonsurvivors was 11 micrograms of antibody per ml as compared to 86 micrograms per ml, for the survivors, and this was highly or statistically significant and correlated very well with protection.

Perhaps more interestingly, effective dose of 50 and 95 calculations, values were determined, and, for instance, it was determined that a circulating quantity of 420 micrograms of the antibody, 420 micrograms per ml provided effective protection to a 95 percent level.

So, ultimately, the goal, of course, would

be to find a similar kind of level in vaccinated humans.

The problem, of course, with this type of assay is it doesn't account for strains of Y. pestis that are F-1 negative, yet retain nearly full virulence, and have been shown to overcome F-1-based immunity.

As a consequence of this, there are several in vitro correlates of immunity to both F-1 positive and F-1 negative Y. pestis strains are in the process of being examined and developed.

As the alternate non-F-1 antigen selected, of course, V was our first choice, as has been mentioned over and over again at this point. It is an essential virulence factor, it is highly immunogenic, and can confer protection, anti-V antibody can confer protection by passive vaccination and the antigen by active immunization.

This is a diagram based mainly on one of the protective monoclonal antibodies applied by Jim Hill at DSTL and mentioned and discussed by Dr. Williamson, but in any event, Jim Hill developed a

set of monoclonal antibodies with different epitope specificities that were specific for different parts of this 326 amino acid V molecule, and whereas, antibodies directed towards more the N-terminus were found to not be protective in a mouse challenge model.

The passive immunization with these monoclonals did not protect, whereas passive immunization with various ones directed in the region of about 135 or 275 amino acid in that region, such as the 7.3 were found to be protective.

So, these kind of responses would be those that it weren't taken into consideration in developing an in vitro correlate.

As Dr. Williamson mentioned, both USAMRIID and DSTL have been working on competitive ELISAs based around a protective monoclonal antibody directed against V, and in this case, as she mentioned, they have been working with the 7.3.

This is just a very, sort of gross oversimplification of a couple studies, very nice

studies of Dr. Garmody--I might have pronounced that wrong--and Dr. Williamson and coworkers that came out recently in Vaccine where a competitive anti-V ELISA was described, and it just involves a couple studies that were done with an attenuated Salmonella live vaccine that produced recombinant V, and a DNA vaccine plus a booster protein of V, and both studies show that they could elicit partial protection with these vaccines and provided a nice range of sera for being able to use to develop in vitro assay to predict survival or not in ultimately challenged animals.

They had both assays for direct endpoint ELISA titers measuring V antigens specific antibody and a competitive ELISA based on competition of the serum antibody with this protective monoclonal.

However, there was no significant association reported between the titer of the competitive anti-V antibody and survival of these mice. There could be a number of reasons, but anyway we can discuss that later.

So, overall, this has been somewhat of a

challenge to develop solid in vitro correlates, but in the same vein, a competitive ELISA based on competition of a serum antibody with a protective monoclonal anti-V antibody has been worked on at USAMRIID namely by Tran Chanh and coworkers at USAMRIID, and a number of monoclonals directed against V have been made available to these workers, and so far they have identified 5 that produced high ELISA titers of antibody in vitro and also provided protection against lethal challenge of mice in vivo.

These 5 antibodies, well, I show them here, and as I mentioned, they exhibit high anti-V antibody titers in an endpoint ELISA and they can passively protect mice.

This is just a summary of some of the passive experiments. This is the summary of all 5 monoclonals, but basically, they provided approximately 50 percent to two-thirds protection of the animals and positive control gave total protection is rabbit, polyclonal anti-V antibody showed previously to be very protective, whereas,

untreated animals weren't protected.

I failed to mention the model here was immunization, treatment intraperitoneally with the antibody, and then challenged was with 25 LD50 by the aerosol route, so it was a fairly realistic challenge, and as I mentioned, the passive therapy protected against that.

I think that antibody was given 24 hours prior to challenge. I am not positive, but I think that is correct.

One of those antibodies, 141 was selected for use to develop an in vitro competition assay. It is not too interesting.

Also, I am not going to discuss this in detail, just to mention the obvious question, if these antibodies are protective, what is the epitope that they are recognizing. Dr. Chanh and his coworkers are just in the process of examining this question. They are using a protease protection type of assay, but beyond that I can't say too much yet, just to answer the question that is obvious.

I don't need to spend time on that, but it is the same kind of drill here. The plates are in this competition V-based assay. Plates are coated with V, a titer of protective monoclonal antibody is established that will give a sensitive level of detection of whole antibody, and then the samples are diluted out, the standard curve monoclonal antibodies diluted out, and the competing biotinylated monoclonal is added, and so forth, the plate is developed.

So, that was the development of the assay. Now, the investigators are, of course, in the midst of real contesting of this assay with sera from animals that have been immunized with F1-V and subsequently challenged. They collect the pre-challenged sera and assess levels of competitive anti-V antibody, and then correlate that with the ultimate survival.

The only thing interesting about this, this just shows one of the sets of sera that they have examined. These were mice that were immunized subcutaneously, two doses of F1-V, the fusion F1-V

antigen, and then they were challenged subcutaneously with 5 times 10 7 LD50 doses of the CU92 strain.

The four dose groups tested are shown here. This is the dose of the F1-V fusion vaccine, and this just gives the numbers of animals that we were working with.

This is a summary of the results. I will just show you this first. These are the sera from all the survivors, you know, pooled from all those groups, are assessed, and then, similarly, the pre-challenge sera of the nonsurvivors were measured in this assay.

It was found that the mean value of the survivors in terms of again the quantitative level of competing anti-V antibody is 44.6 micrograms per ml as compared to 7.8 micrograms per ml in the nonsurvivors, and this was highly statistically significant and are correlated very well with survival, and gave a predicted effective dose, 50 of 8.2 micrograms per ml of that antibody in serum.

I won't spend too much time now. The

obvious tests there are to perform the fact they are using sera of immunized nonhuman primates, this is in process. We are testing sera generously provided by Dr. Pitt and her coworkers involving the models of the African Greens and the cynomolgus macaques.

The first set of sera, most of the animals in the experiments either lived or died, and the sera aren't appropriate really for trying to assess a correlation between survivors and nonsurvivors, if you have everybody has lived or everybody has died makes it kind of difficult, but more experiments have been done, more sera has been collected, and Dr. Chanh and his workers are very busily assessing the sera.

I can't say a lot about it yet unfortunately, however, I took the data that they did do, they did assay from some of the very early studies where all the animals in one group died and all the animals in the others lived, and I kind of pooled it together.

They took the competing ELISA titers of

these four groups of survivors and nonsurvivors, and it does appear that we are getting a similar trend in the nonhuman primates that we saw with the mice in that the survivors will indeed have a significantly enhanced level of competing anti-V antibody compared to the nonsurvivors.

Now, in addition to the antibody-based assay, we have been looking at sort of a more functional assay of anti-V activity to provide an additional correlate, in vitro correlate, and we have been examining assays for antibody based on neutralization of macrophage cytotoxicity.

As nicely described by Dr. Bliska, at least in the later stages of infection with a virulent Y. pestis, the organisms in vivo resist phagocytosis and they cause an infection that is mainly extracellular.

In vitro, this can be modeled by appropriate pregrowth of Yersinia pestis will put them in a state that they resist phagocytosis and are cytotoxic for macrophages. We wanted to see if we could develop this model as the basis of an

additional cell-based in vitro correlate.

So, the question was can antibodies that protect against pestis in vivo neutralize this in vitro macrophage cytotoxicity assay, and, if so, what is the role of V and anti-V in all of this, and as you have heard and I won't belabor the point, V is required for the type III secretionmediated translocation of the cytotoxic Yops. This is just a nice, very simple diagram that was published in an article in 1999 by Drs. Field and Straley, and this just shows the close contact that is required for this process of the pestis inducing the cytotoxicity of macrophage, direct contact between the organism and cell stimulates the production of the Yops and their secretion and translocation into the target cell.

As you can see, what has been called sometimes the injectozone, which is the needle through which the Yops are translocated, it appears that V is the special component of this, so it is essential in the actual delivery of the cytotoxic Yops.

V, of course, has multiple roles and I won't discuss any of the rest of this further, but if V is so essential in the translocation of the cytotoxic Yops, the question was can antibody prevent the whole cytotoxicity.

We tested this. Steve Weeks was the postdoc in my lab, and he developed a nice macrophage assay to examine these questions, and the initial assays were done just simply looking at LD8 release, a terminal marker of necrosis, cytotoxicity, and cell death, and he found that when macrophages were grown, well, when Yersinia pestis was grown in vitro for 2 hours at 37 degrees, and then incubated, pretreated or incubated, the cells were incubated with normal rabbit serum, and these organisms were then used to infect cell cultures, there was no effect of the normal serum on the cytotoxic activity, that indeed the Y. pestis was cytotoxic for the macrophages and killed them, however, the organisms were similarly pregrown in vitro and then incubated with the rabbit anti-V antibody.

Then, the mixture then used to infect macrophages, that this treatment seemed to ablate the cytotoxicity. The same effects were seen with the isolated FAB fragments of the antibody, suggesting that the protection was just not merely due to recognition by the FC portion of the FC receptors of the cell.

We wanted to know if the death of the macrophages was due to necrosis or perhaps might be a reflection of an apoptotic or programmed cell death phenomenon, so we, instead of measuring LDH or besides measuring LDH release, we also did assays to measure the caspase enzymes. Caspase enzymes are proteases that are made specifically only during apoptosis, programmed cell death, and the caspase-3 enzyme is one that is made early in the process of the cell going through this death phase.

We wanted to see if this marker could correlate with what we have seen with the LDH release, and basically it did. When you pregrew the bacteria, the pestis, the fully virulent

organisms or the pgm-minus organism, the same thing, they effectively cause the increased production of greater levels of caspase than is seen in uninfected cultures, so it seems to induce an apoptotic type death pathway.

As expected, uninfected cultures--I didn't show this--cultures infected with the organism that was cured on its virulence, also when there was no cytotoxicity, and organisms with a mutation in a critical translocation protein YopD also were ineffective.

After developing the assay, we wanted to test whether it was predictive in these animal studies. The question was: Can serum macrophage cytotoxicity neutralizing activity from immunized animals serve as a quantifiable predictor of protective immunity?

The first tests were done with mice immunized with F1-V, similar to the set that I mentioned for the competitive ELISA. These animals received 2 doses subcutaneously with different doses of F1-V fusion vaccine, and then were

challenged subq, and we tested the association between survival and cytotoxicity neutralizing activity of the antibody and also the effect of the vaccine dose.

This just gives the results, sera from individual mice. The mice were immunized. During the course of immunization, from day zero up to just before challenge, sera were collected from the animals to see if there was sort of a development of neutralizing antibody or development of the anti-V antibody.

We took all these sera from each mouse and titrated each of them, tested different dilutions, and then used the sera in the in vitro assay to incubate with the organisms prior to the infection of the macrophage cultures with the organisms.

This shows the data for one mouse that ultimately lived after immunization. As you can see, over time, from day zero to just prior to challenge, there was an increasing development of antibody that was better with time able to neutralize macrophage cytotoxicity as indicated by

the quantity of caspase enzyme that was detected, so that quantity of that death-related enzyme was dropped with time as more antibody--the data is not shown here, but also the direct ELISA titers of the anti-V antibody increased with time.

As they increased with time, the amount of neutralizing activity also did. It just shows two different dilutions. In contrast, these are the set of sera from an animal that ultimately died, and as you can see, there is no real pattern to the development, no real evidence that cytotoxicity neutralizing activity has developed.

We submitted the results of the studies of all the animals and all these titrations for statistical analysis, and we found the statistical outcome was that the vaccine dose together with the decrease in serum caspase from days 1 to 56, just prior to challenge, correlated well with survival.

This is sort of shown graphically here and that the change in caspase levels over time during the process of immunization is plotted at the bottom. Negative values mean that there is

increasing neutralization of the cytotoxicity, which means decreasing levels of the caspase. Zero or positive values indicate that there is no effective neutralization, and you can see this represents all the mice from the 0.1 microgram dose group of F1-V.

This gives the probability of survival from zero to 100 percent. In animals that were shown later to survive, they had negative values in that they showed a large drop in caspase levels during the course of immunization whereas the animals that ultimately died did fail to develop neutralizing antibody.

We did similar studies with animals that were just vaccinated with a single dose of F1-V. This just shows the groups that we had. There were 7 vaccine dose groups. They all received one dose of vaccine from 30 micrograms to zero on day zero, and then challenged on day 28.

This gives the summary of the results. Again, animals immunized with one dose, we found that the mean cytotoxicity-neutralizing value of

the sera of the survivors was highly significantly greater than that of the nonsurvivors and quite predictive of protection.

This was the first set of reagents we have been able to test where the macrophage cytotoxicity assay by itself was a marker predictive of detection. It wasn't dependent on vaccine dose or anything. It was independently predictive of infection.

Remaining challenges. Of course, we want to kind of verify the usefulness of this assay using sera from primates that have been immunized and we are, as I mentioned, in the process of trying to analyze such sera now.

The ultimate goal would be to determine a level of serum in vitro neutralizing activity that predicts protection in both encapsulated and nonencapsulated organisms.

So, just to summarize, promising correlate assays of F1 and V antibody activities are being developed, however, a thoroughly tested correlate assay for immunity to plague has yet to be defined.

This will require for both the competitive ELISAs again rigorous tests with sera from nonhuman primates and the same requirements for macrophage cytotoxicity assay. Decide on a very definitive and rugged standardized assay and then complete tests with nonhuman primates.

I won't go into this, but in the event the macrophage caspase-based enzymes fail to provide a very good correlate of immunity, we are also at the same time examining other markers of cytotoxicity that cover the whole range of the apoptosis cascade from very early events in apoptosis to the terminal necrosis.

We are in the process of looking at a number of different assays plus we are also, in addition to mouse cells as a macrophage cell type, we are looking, examining whether human-derived cells might be more better predictive, their responses might be more predictive, for instance, of the activity you would get with the nonhuman primate sera, so we are sort of actively looking at this.

There have been contributors over the years to this project. Tran Chanh, of course, contributed some data to this presentation, and he and Sylvia have provided numerous mouse sera, as well as monoclonal antibodies.

Steve Weeks, a postdoc in my lab, was the first to develop the macrophage in vitro assays we had. Jim Hill provides monoclonal antibodies, such as the 7.3. Jackie Bashaw is currently working very hard on these assays in my lab. Kelly Rea has been previously associated with that work, and then a number of people have contributed animal sera from their vaccine studies, Jeff Adamovicz, Gerry Andrews, Louise, Chris Bolt.

That's it, the end. [Applause.]

DR. QUINN: We do have some time for questions.

DR. MIZEL: Steve Mizel, Wake Forest. What form did you immunize with F1-V, did you have alhydrogel?

DR. WELKOS: I believe it was always formulated in alhydrogel.

DR. MIZEL: I have another question which relates to testing in these animal models. We are making up antigens that are really not done in a GMP facility. So, is it possible, do you check for endotoxin levels and bacterial DNA, things like that, so that when we transition to humans at some point that we are not--and we are making fewer preparations that can go into humans, that we might see different results?

DR. WELKOS: I believe endotoxin levels have been checked. DNA, yes, in some instances, because there are studies that are kind of pre-GLP at this point in time, can anybody else from my place comment on that?

I can't give you numbers, but these kind of tests are being done because some of this work is at nearly GLP stage.

DR. WILLIAMSON: I just wonder whether you can say anything about how these assays read out between mouse and nonhuman primate, are you getting very similar results in the nonhuman primates?

DR. WELKOS: Are you talking about the

competitive?

DR. WILLIAMSON: Yes.

DR. WELKOS: Just that one graph I showed that suggested that it was a promising indicator that the primates that went on to survive were giving higher titers of competing anti-V antibody than the nonsurvivors, but like I said, we are just now collecting some data which provide a nice range of sera from survivors and nonsurvivors, which we have been needing, and they are being tested, but it seems promising, but beyond that, I can't say.

It will be very nice when they have characterized the epitopes, the specificity of some of these protective monoclonals, and more can also be said at that point I think.

DR. WILLIAMSON: Another quick question is then do you see a difference between the African Green and the cynomolgus model in the competitive ELISA?

DR. WELKOS: I don't know. I don't have that information.

DR. BLISKA: My questions are about the

cytotoxicity assay. It looks like it's working great. I was curious about a couple of details.

When you are using serum, have you ruled out that there is complement-mediated killing of the bacteria during the cytotoxicity stage, for example?

DR. WELKOS: Not directly.

DR. BLISKA: I think the organisms are resistant, but I was just curious.

DR. WELKOS: No, I am sorry, we haven't directly addressed that question that I can think of.

DR. BLISKA: The other issue was I have noticed--and maybe you have switched to using Yersinia pseudotuberculosis--and I noticed that Dr. Williamson had also. I am wondering, is there a reason for that, is it just more reproducible?

DR. WELKOS: It works also with pestis, so we can do it under BL2 conditions. We use a straight test, pgm-minus and Pla-minus, highly attenuated, but Jim Hill clued us in to the pseudotuberculosis, the strain that is mutated for

its own V and is transformed with this nice expression plasmid to PTRCB plasmid, that I think Dr. Forrestburg originally isolated, that produces a nice quantity of V, and it just gives nice, cleaner results. It gives better cytotoxicity sometimes in our controls. We always have a set of controls, you know, untreated to make sure that we are killing the cells.

They seem to give comparable results with the pestis, but we have just gone with the Y.ptb for now just because it is easier to handle, you know, better, easier, cleaner results.

DR. BLISKA: The last issue is you mentioned that sometimes in this, I would say you get some translocation of the Yops even with neutralizing antibodies, so I was just curious if you considered measuring cytokine productions for something in addition to apoptosis, it might be another reflection of a neutralizing.

DR. WELKOS: That would be an excellent thing to do. The only thing we had done was try to see if anti-V antibody would kind of neutralize the

stimulation of IL-10, and that was kind of a bust, but your suggestion is well taken. That is something we should consider.

DR. QUINN: Last question.

DR. PERRY: Bob Perry, University of Kentucky.

Just to quickly answer Jim's questions about complement-mediated killing, they are resistant in the absence of specific antibody, and I think Bob Brubaker had a paper that showed that it was probably due to the short LPS, no antigen side chain was involved in that.

But in the absence of a specific antibody, they are resistant, so it is not a problem with the assay.

DR. QUINN: Very good. If there are no more questions, then, we will close the session and thank the speakers once more for their presentations.

[Applause.]
[Luncheon recess taken at 12:20 p.m.]

AFTERNOON SESSION

[1:40 p.m.]

DR. MEYSICK: We will get started for the next session.

Session 3: Human Disease and Relevant Animal Models

Moderator: Dr. C. Richard Lyons

The next session is Human Disease and Relevant Animal Models, and the moderator for this session is Dr. Rick Lyons from the University of New Mexico.

Rick.

DR. LYONS: Thanks, Karen.

This session, we will take a look at the epidemiology of human disease and how the animal models relate to that.

The first speaker is Jacob Kool from CDC, Fort Collins, and he will be talking on plague epidemiology and human disease.

Plague Epidemiology and Human Disease

Dr. Jacob Kool

DR. KOOL: I would like to thank the organizing committee for inviting me to this very

interesting workshop.

I will be the one I guess giving the background talk about clinical aspects of the disease and epidemiology, but I am especially excited about the opportunity to tell you about the CDC clinical trials that we are currently doing.

We are evaluating drugs and diagnostics in Madagascar and in Uganda. I wonder if those drugs might also be used for a vaccine trial.

I developed a slight cough on my way back from Madagascar, I just came back a few days ago.

I hope it is not a slight case of pneumonic plague, but I have to apologize because I didn't have time to submit my handouts in time to be included in your handout.

In this presentation, I will talk about the epidemiology of plague in the world, in the United States, and the implications of bioterrorism. I will talk about clinical aspects of plague, of course, naturally occurring plague with an emphasis on pneumonic plague.

Karen Meysick suggested I should bring a

lot of x-rays. Now, it is pretty hard to get x-rays of pneumonic plague, but I have done my best.

At the end, I would like to mention a few field sites in Uganda and Madagascar.

You have already seen this picture, the global distribution of plague. The red areas are actually the interesting areas where we think there are still sylvatic, endemic foci of plague. It is the western U.S. where there are only about one or two cases a year now.

South America, Asia, there are probably still a lot of cases in southern China, we don't hear a lot about them. They don't always get reported. In fact, more than half of all cases, about 80 percent of all cases are reported from this area, eastern Africa and Madagascar. Up to a few years ago, Madagascar reported about 50 percent of all cases of plague in the world through WHO.

In the United States, as you all know, plague was first imported into the U.S. in 1899. It first caused outbreaks in San Francisco in the Bay area, and in Los Angeles in '24, and then it

suddenly seemed to almost disappear until it shot back up in the sixties and especially peaked in the mid-eighties.

Maybe in this period in between, these outbreaks usually were transmitted or propagated by urban rats, and these outbreaks in these cases are usually associated with wild rodents in the western plains in U.S., so perhaps these rodents needed these years here to get infected, to establish the infection.

So, nowadays, most cases of plague occur in New Mexico, and they are usually sporadic cases of bubonic plague associated with rodents like prairie dogs.

This is the way plague is transmitted. Our plague ecologist gave this slide to me. Here is a picture of a prairie dog. There is this epizootic cycle of prairie dogs and their fleas, and occasionally, very rarely really, it gets transmitted to humans, for example, when a human passes a prairie dog colony, and the dogs, when they are dead, the fleas will look for another

host.

What happens a lot, too, is that other animals, for example, cats who are hunting for prairie dogs get infected, and cats can develop plague especially pneumonic plague. The only cases of pneumonic plague that we see nowadays are usually cat associated.

So, when the case of bubonic plague turns pneumonic, then, you can have the cycle of transmission among persons, of course, and these domesticated animals and mice and rats theoretically can also sustain a cycle, but this is very rare nowadays.

This is a typical picture. In 2002, there were two cases, a couple who traveled from their home, this home in New Mexico to New York City and developed plague while they were in New York City. You can see that this is a typical habitat of prairie dogs, and there is clearly a short
interface between humans and wild rodents in this type of dwelling.

Plague occurs mostly in the summer in the

United States. This is the clinical presentation that we see in the United States. Over 80 percent is bubonic plague. Then, the next chunk is septicemic plague, and only about 2 percent are called pneumonic. This is the primary presentation. Of course, there are some pneumonic cases among those bubonic plague cases, secondary pneumonic cases.

The bioweapon potential of plague, as we mentioned before, it is thought that Yersinia pestis was recognized by the former Soviet Union for aerosol delivery. Theoretically, it can be engineered for antimicrobial resistance or virulence. F1 deficiency, I am told is quite easy to get into the bacteria, and this would have implications for vaccine development, but also for diagnostics. Most of our diagnostics are based on detecting the F1 antigen.

Theoretically, maybe lyophilized formulations could be used as a weapon. We don't know what will happen in the environment if after releasing over a city, if it will establish itself

among the urban population again.

This is the only Category A bioterrorist agent that can also be transmitted from one person to another.

So, in 1970, WHO called in an expert panel, and they estimated that if 50 kilograms of Yersinia pestis would be released over a city of about 5 million, this could cause about 150,000 cases, more than 30,000 deaths, hospitalization for up to 100,000 people to a secondary spread, they thought might affect another half million people with up to 100,000 deaths.

This is an old picture of typical bubonic plague, typical bubo. This must be the place where they tested the aspirate, where the blood is.

Here is a picture of a septicemic plague case. All you can show really is a very sick, obtunded patient.

Pneumonic plague. As we mentioned before, there are two forms of pneumonic plague. Secondary pneumonic plague is what we see normally. It is caused by hematogenous spread of the bacteria from

a bubo or from blood in the case of septicemic plague to the lungs.

Primary pneumonic plague, of course, is caused by direct infection of the lungs, and this is the disease that we are really interested in today, because that is caused by terrorism, as well.

Primary pneumonic plague, what we know about primary pneumonic plague is mostly from historical accounts especially the large outbreaks in Manchuria in 1910 and 1920 where, in total, about 76,000 people died of primary pneumonic plague. Since then, there have been only very small outbreaks.

We know that it has a very short incubation time, probably 2 to 4 days, and the range may be between 1 and 6 days, but there are a lot of questions about those historical accounts, about determining the date of onset with those patients.

It typically has an acute fulminating course characterized by a systemic inflammatory

response syndrome with disseminated intravascular coagulation, ARDS, so they require intensive support, and just a few cases could easily overwhelm the capacity of the health care system.

Mortality is 100 percent. People usually die within 3 to 6 days after onset if they are not treated early, and that means it is clearly necessary to give the first dose of antibiotics within 20 hours of onset, and that is quite a challenge.

In the United States, as I mentioned, there have been some outbreaks of primary pneumonic plague. The only cases really of human-to-human transmission were in 1919 and in 1924 in Oakland and in San Francisco. Since 1925, there has been no human-to-human transmission of plague in the United States.

There have been 8 cases of primary pneumonic plague. Six of those were associated with cats. Most of them were veterinarians who were treating a cat with pneumonic plague. One was associated with a laboratory accident. Someone was

centrifuging Yersinia, and the vial broke, and one remains unknown.

I tried to give a description of what does primary pneumonic plague look like, and I have to go to very old sources, Wu Lien-Teh from Manchuria in 1926, Pollitzer, Tom Butler in Vietnam.

What they seem to describe as a typical case is an initial noninfectious stage which might last several hours, up to about 24 hours. Wu Lien-Teh calls it a noninfectious stage because he noticed that hardly any of these patients ever contaminated other people during this stage.

This stage is characterized by a sudden onset of malaise, chills, severe headache. There is increased respiratory and heart rates, and during this stage, the temperature rises steadily.

After several hours, you will see a dry cough develop which becomes progressively productive, but even the sputum still doesn't contain many plague bacilli. It was usually very hard to find any bacteria in the sputum.

This might continue for hours up to a few

days even, and in the final stage, this means a few hours before they die, maybe only one hour before they die, the patient will have bright red sputum, and if you look at that under the microscope, you find many plague bacilli in almost pure culture, as they describe it. These are the patients that are very infectious.

So, it is kind of hard to recognize a case of pneumonic plague in the early stages, and in these days, the patients should rarely actually progress to this stage here. This is only when patients are not treated with antibiotics.

Here are some pictures from the outbreaks in Manchuria. Here are two cases of pneumonic plague. This is a patient in the early stage, and this is a patient in the final stage, just before death I guess.

Here you see a patient with blood-stained bed linen who is coughing up red sputum. Here is one household, everybody, all the dead people in one household. There were lots of pictures like this.

Here are some more pictures of pneumonic cases. You see how the health care workers protected themselves with masks. These patients were examined in the open air.

The only picture that I could find, the only x-ray of the primary pneumonic plague case in the United States is this one. This was a 22-year-old male in California. I believe it was the mid-eighties. It is not clear where he got his infection, but he started to feel ill on a Friday, and he even reported to work on Monday, and only on day 5, on Wednesday, is when he was brought into the hospital moribund, he was very ill, in severe respiratory distress.

This is the x-ray that was made then. You can see a large infiltrate in the right lung. Only at 12 hours later, the patient looks like this. He developed adult respiratory distress syndrome, and he died within two days of hospitalization in spite of mechanical ventilation.

Here is his hand. This was before we gave this disease the connotation of the Black Death.

This necrosis occurs, not just in the hands and feet, but in all organs in the body.

Here is another. I apologize for the quality of this picture. There is an interesting article by Alsifom in 1981, but all these cases are secondary pneumonic cases.

You see, in this case, a large number of deaths. Only 12 hours later, this has become much worse with bilateral infiltrates, diffuse bilateral infiltrates.

This patient, you see left pleural effusion, and this was actually made a few weeks after recovery. He still has a cavitary lesion.

This patient shows bilateral pulmonary parenchymal infiltrates.

This is a case of bubonic plague that does not have pneumonia. This whole picture is actually caused by the DIC, not by infection of the lungs. So, an x-ray doesn't always tell you if it is pneumonic plague or not.

Treatment of plague is parenteral. It is done with these antibiotics - streptomycin,

gentamicin, doxycycline, ciprofloxacin. Gentamicin and ciprofloxacin have not been FDA approved although they are part of the national pharmaceutical stockpile. Prophylaxis can be done with co-trimoxazole.

Person-to-person transmission. Contrary to what many people believe, plague is not very contagious. The risk is not as big as people think. The last time this happened in the U.S., as I said, was 1924, and it only happens in very close contacts. You have to be closer than at least 2 meters, and the surgical mask is probably protective. This is what health care workers in Manchuria used, and it was quite effective. It was made of cotton.

Like I said, they are only infective in the later stages, and after one day of antimicrobial therapy, patients are not infectious anymore.

So, I would like to show you a bit of our field sites in Africa. We are doing field sites in Uganda and in Madagascar. Our project consists of

two parts. One is to determine safety and effectiveness of gentamicin. In Uganda, we compare it to doxycycline. In Madagascar, we compare it to streptomycin because those are the nationally used regimens.

We also take this opportunity to evaluate newly developed rapid diagnostic tests, dipstick kind of tests. My colleague, Marty Schriefer is here. If you have any questions about this part, he will be happy to answer it.

We are evaluating four brands of dipsticks, and they are all based on detecting the F1 antigen. This study, by the way, is funded by FDA/CDER.

These are the four diagnostic tests with dipsticks. All of them were originally developed by the U.S. military, but this one was taken over by the Institut Pasteur in Madagascar, and they are already using it in that country. These three are newly developed, and we are evaluating those together with the Institut Pasteur dipstick.

For now, they have only been approved for

nonhuman use. We hope to change that. They are showing very good results so far.

These are the countries - Uganda and Madagascar, and we found women to collaborate with us on this clinical trial. We have been preparing for this study for about two years now. We had to completely renovate and equip central laboratories in each country that were close to the plague endemic areas.

The field sites where patients actually come to the clinic had to be equipped also with colorimeters to test kidney function, and we did all kinds of other things, electricity, refrigerators, communications. We had to get vehicles to transport specimens and to transport patients, and we have hired and trained many field staff. IRB approvals were quite a challenge, but we got it approved. Accounting is also important in these countries. Uganda ranks I think number 5 among the most corrupt countries in the world.

In Uganda, plague tends to occur in these highlands here, at the border with the Congo in

northwestern Uganda. This is the West Nile region.

Our field sites. We have our central laboratory located in Arua, the largest town of the West Nile region, and we have 14 field sites along the Congo border where we expect to see cases of plague.

Uganda sees about between 200 and 500 cases per year in that small area. Cases have a seasonality. They occur mostly between September and December. This is after the harvest when people bring their harvest into the house, and the rats follow the harvest, and they bring plague into the house.

Here are some pictures of rural clinics in Uganda. I really wanted to put your attention to this one. The plague isolation ward of Agiermach.

This is a plague case. A young boy who had bubonic plague last year.

This is the laboratory. It is an area that had been ravaged by civil war several times, the last time about 15 years ago, this building was sacked. We were donated this building, and we

renovated the whole thing, and it now looks like this.

This is when our equipment arrived. They brought it in a big container. There is no crane, so they had to tie a rope to a tree and then drive the truck out underneath it. This just gives you idea of the remoteness. Fortunately, they had taken out the equipment before they tried this, so this is what happened.

We were lucky. This is Marty Schriefer. He is sitting right there. We were lucky that he was standing on this side, but no problem, we just roll it back, and it is now our storage shack.

I will show you some pictures of Madagascar. This is rural Madagascar. They have not yet invented the chimney. The reason why in Madagascar there still are small outbreaks of pneumonic plague, family outbreaks of pneumonic plague, at night they hermetically close their doors and windows. I think it has something to do with a fear of ghosts, maybe also to keep the warmth in. As you see, there are no chimneys, so

it is really a great place to get any respiratory infection.

Suspected cases of plague in Madagascar, as reported to WHO, they have reported up to 3,000 cases in the mid-nineties, but this has gone down, and confirmed cases have always been quite low. Because of the distances, it is really hard to confirm any cases microbiologically.

The cases that I have gone down for, because they have started to use the dipstick test, and they were able to show that many of these cases actually were not plague. So, now there are several hundred cases a year in Madagascar. Lethality is now about 20 percent.

Here is a typical bubonic plague, an early bubo without pus. They regularly see pneumonic cases, like I said, but these are already recovering or recovered almost.

Our field sites are in the highlands. This red area is where plague occurs in the highlands mostly. We have chosen this small area where the incidence has been the greatest in the

last five years, and we have equipped 10 rural clinics and the Central Plague Hospital in the capital of Atonaria [ph].

This is the Central Plague Hospital. We built our lab there completed by a safety cabinet and freezer and everything. The rural clinics have been equipped with solar panels. Here, you can see someone working with a centrifuge that we gave him on the first samples from the first case of this season.

Here is a colorimeter that he is going to use to determine creatinine for renal function.

This is our first case. She was brought in severely dehydrated after a ride over this road actually for six hours in a wheelbarrow. This is the type of isolation that these people live in. This is actually her village. We went out there two days ago. This was e-mailed to me last night. It is only a hamlet of about five of six houses.

The dipstick test did very well on this patient. I haven't heard how the patient is doing now, but we expect that she will recover.

This is my last slide. So, our timeline for our clinical trials are to go--we have just started, like I said, this October--we will go for two seasons. So, the project is projected to end in the spring of 2006. After that, we think it will be a waste not to keep on using these laboratories in these field sites, so we hope that we can find a way to continue this work. Maybe we can develop novel methods for control among rodents and fleas. Maybe we can follow up our clinical trial to test fluoroquinolone, and who knows, maybe these sites are useful for vaccine evaluation, as well.

Thank you for your attention.

[Applause.]

DR. LYONS: In order to stay on schedule, we probably have time for one or two questions.

DR. FROTHINGHAM: Rich Frothingham, Duke University.

You alluded to the importation of plague to the Americas in San Francisco, I think in the latter part of the 19th century. I wanted to pose

to you the question that I get all the time, which is, of course, this would never happen, my mice are never going to get loose, but the question is if my mice get loose, they will be consumed by some predator pretty quickly, what is to stop the development of a new sylvatic focus, and why is it that these foci develop in certain places.

DR. KOOL: That is a very good question. I don't think I have an absolute answer to that, because at the same time when these rats were introduced into San Francisco Harbor, of course, they came to New York, as well, and to Houston, everywhere where there is a port.

So, there must have been something about the western U.S. that is more friendly to plague, to developing a sylvatic focus. Of course, in those days, hygiene was much worse, there was much more interface between humans and rodents in their houses, so, yes, the big question is, could it establish itself again among the urban rodent population. I don't know the answer to that.

DR. McINNES: Pamela McInnes, NIAID.

I am sorry, Jacob, if you mentioned this, I didn't get the actual design of your clinical trial, your interventions. I didn't get that.

DR. KOOL: I didn't have much time to go into that in detail, but what we do is we compared treatment with gentamicin to the treatment that is normally used in those countries, to the national standard, approved standard of care.

In Uganda, the approved standard of care is doxycycline, or tetracyclines in general, and in Madagascar, they still use streptomycin, intramuscular injections. So, a patient with plague who comes in with suspected plague is randomized into one of two treatment arms. They either receive gentamicin for 7 days or they receive the other drug, the national drug, and then we follow them equally.

DR. LYONS: I just have one question. If anybody in the crowd had a diagnostic, however, are these sera at all available, or are you going to stockpile them, so that people could have access to the infected sera to test against gold standards?

DR. KOOL: Yes, we thought this was the great opportunity to get new specimens from plague patients, so we do ask for the patient's consent to keep their serum and their aspirates, and we plan to transport them back to the United States.

DR. LYONS: Great.

Thank you very much.

[Applause.]

DR. LYONS: The next speaker will be Pat Worsham from USAMRIID on small animal models of plague.

Pat.

Small Animal Models of Plague

Dr. Patricia Worsham

DR. WORSHAM: I was asked to concentrate today on historical perspective on small animal models for plague and to try to tie that in with the animal models that we are using predominantly today.

One of the very first animal modelers was Yersin himself. He isolated a live attenuated vaccine strain which he found was virulent in rats,

but not in five species of macaques.

He was quite interested in determining which species most closely reflected that of the human, so he identified a human volunteer, in this case himself, and he injected himself, not like Haffkine with a killed vaccine, but with a live vaccine that he hoped was attenuated.

Luckily, he survived this, he had only transient fever, and he declared after this that he believed that the susceptibility of the macaque more closely resembled that of man than of the rat. Not something we can do in today's environment, but interesting nonetheless.

A number of small animal models have been explored. The most common are the mouse, the guinea pig, and to a lesser extent, the rat. Other models have included ground squirrels, rock squirrels, the multimammate mouse in South Africa, various other rodents, lagomorphs, and domestic cats, but there are inherent difficulties in comparing these models.

First of all, historically, there has been

very little consistency in the strains open for study and the strain of animal chosen for study, or the source that the animal was obtained from. In fact, some of these animals were obtained from the wild historically.

There is little consistency in the way the challenge inocula are prepared to the way that experiments are conducted, so it is very hard to compare experimental studies because of this.

The really consistent thing with all of these is that the predominant antigen that has been looked at over the years has been the F1 capsular antigen which you have already hear about today.

Perhaps the best characterized model is that of the mouse. It has certainly been the most utilized. It's an accepted model of bubonic and pneumonic plague. The pathology for the most part resembled that of human disease. It is desirable, obviously, in terms of handling, space, and expense. They are small, inexpensive animals.

You can have a lot of them, and it is a well-established
del for both active and passive

immunization that has been alluded to by other people earlier. It is also useful for nontraditional or modern vaccine strategies.

e LD50 subq is from 1 to 10 CFU and, by aerosol, from 10

:o 105. This is inhaled dose.

Also, the availability of different mouse strains and our knowledge of mouse genetics allows us to explore the role of various components of the immune system in both innate resistance and acquired immunity, and Diane has already alluded to that, as well.

In 1949, K.F. Meyer described the disease progression in mice challenged parenterally with Yersinia pestis, and it is not inconsistent with what we see in clinical cases of bubonic plague.

Fairly soon after exposure of the animal to the organism, they are carried to the regional lymph nodes, transferred to the thoracic duct and the bloodstream. This low grade bacteremia may go on for several hours, it seeds the liver, the spleen, and bone marrow in mice, and after replication of these organisms, there is a terminal

heavier bacteremia.

A number of methodologies have been used to look at what is hoped to be a model of pneumonic plague in the mouse. Small particle aerosols induce primary pneumonic plague. This has been shown pretty consistently.

In many cases, there have been aerosols used which give variable particle size. In some cases you get disease that is characterized, rather than primary pneumonic plague, it is characterized by cervical bubos and septicemia, so it is a different disease process caused by these larger particle aerosols. Pneumonic disease has also been reported in intranasal installation, but only about 10 percent of the inoculum actually reaches the lungs in this case.

There have been a lot of live attenuated vaccines evaluated in the mouse. The results are quite dependent on the type of attenuation that is present in the strains, but some good protection has been demonstrated. There is residual virulence in some vaccine strains, and this has been a

problem over the years especially in the early days when the lesions in the attenuated strains were not really genetically defined.

Inbred mice exhibit varying levels of sensitivity to attenuated strains, and many of these attenuated strains have been, as EV76 is, pgm-minus. Some of them have other lesions, as well, and every strain of EV76 is different from every other strain of EV76, so it is also very hard to go over that part of the literature.

There are reports that CBA mice are more resistant to attenuated strains than C57 Black 6 mice.

Over the years, starting with Meyer basically, the mouse has been used to look at the passive protection model. Meyer used this to evaluate the response of human volunteers to potential plague vaccines, and this has already been discussed to a certain extent, but basically, the mouse is given I.V. the sera in question, and in about 30 minutes, the mouse is challenged over the right inguinal node with about 1,500 CFU of

Yersinia pestis.

There are some really strict standards to do the Mouse Protection Index correctly. Each time the test is done, the LD50 has to be repeated for that inoculum, and the LD50 should be no more than 12 CFU.

The organisms are monitored for 14 days, and the MPI is calculated. It's the percentage of mice dead during that period over the mean time to death. So, as this number gets larger as more mice die, in the meantime, the death is small, that number is very large, and that is not a good protective index.

But as you get more mice through monitoring, and the ones that do die take longer to die, the protective index gets lower, and that is an indication of protection.

There have been some recent demonstrations of vaccine efficacy in mice. One is the fusion protein of F1 and V, and you have heard a lot about those V antigens by now. This is actually a fusion of the two proteins expressed together, that was

originally made by Dave Heath, who is in the audience.

This, combined with alhydrogel, gives excellent protection against parenteral or aerosol challenge in Swiss-Webster outbred mice, and it protects, and this is very important against both F1-positive and F1-negative strains, because F1-negative strains do obtain virulence in the mouse model and in the nonhuman primate model.

Now, the study I just discussed used outbred mice. This is one of Diane's studies here that she already discussed, but just to go through briefly, they looked at inbred mouse strains. All of them responded well with good IgG1 titers.

They used alhydrogel as an adjuvant, so it was a Th2 type response, and this is an interesting point that I had not considered before, and that is that there is a problem with keeping male mice long term because of aggression, and this may affect the results of studies if male mice are used especially long term, but they did point that titers were maintained well in female mice.

DNA vaccines have been used in the mouse model. There were some early reports that a prime-boost approach worked well in inbred mice, but not in outbred mice. These immunizations were done IM.

More recent studies indicate that the gene gun might be a better approach as an immunization route and that combining it with a vector that targets expression to cytosol may be more successful, but to my knowledge, most of these later studies have been done with inbred strain mice, as well, so we really haven't resolved this issue.

The guinea pig is also the historical model of plague. It is quite sensitive to Yersinia pestis, but historically, there is reported to have been a seasonal resistance to infection, that is, during certain years, at certain times of the winter, the guinea pigs were not as sensitive to infection.

Some of it may be circumstantial. There was a belief among some early plague researchers that certain lots of guinea pigs were more

sensitive or resistant to plague in general, and that perhaps using a more inbred strain of guinea pig and a more consistent source would help their result.

It is known that F1 capsule is an important virulence factor in this model, although F1-negative strains are attenuated in the guinea pig, they are not in the mouse significantly, and they are not in nonhuman primates, so this is a difference between the guinea pig and other models.

For the most part, the disease resulting from subcutaneous infection is similar to that seen in mice.

The very early aerosol models are described in the guinea pig around the turn of the century, the previous century. Culture suspensions were described as being sprayed in the air, so these are probably multi-size particles, and for the most part, it did not really induce true pneumonic plague.

The necropsy showed cervical and laryngeal edema, cervical buboes, septicemia, and hemorrhage

of the intestinal wall. So, this is probably more of a pharyngeal plague being produced. In some cases, there was evidence of secondary lung infection, but not primary.

There were additional reports of intratracheal installation of organisms that could cause pneumonic plague although the percentage of animals that actually acquired true pneumonic disease from this was not clear.

Intranasal models were also discussed in the literature. These animals were anesthetized and about 10 percent of these organisms actually make it down to the lung, and it is interesting to note that some guinea pigs did transmit the infection to control cagemates during these studies, but it is not clear whether it was pneumonic transfer or other methods.

There were some nice studies done by Druett in 1956 showing that the particle size affected the course of disease in guinea pigs. Guinea pig respiratory tract apparently does not allow particles greater than 4 microns to reach the

lungs.

Particles less than 1 micron initiate a bronchopneumonia, very characteristic of a few in pneumonic plague, and the larger particles deposit in the upper airways, and that is where you start to find the cervical nodes and septicemia, but not any primary pneumonia.

The guinea pigs, this is a more modern study. This is one that Sue Welkos put out a few years ago. The aerosol LD50 or type strain Colorado 92 is similar in the guinea pig to that of mice, but unlike the mouse, F1-negative strains were attenuated in the guinea pig model, and what Sue found was that parenteral infection was protracted and often not dose related, which is not a good thing in an animal model.

In general, what has been found is that guinea pigs respond better to live attenuated vaccines than to subunit vaccines. You can enhance the response to subunit vaccines by giving them a very large antigenic mass, much larger than other experimental models or by addition of oil-based

adjuvants, which are not really considered to be ideal in today's environment.

Passive protection of guinea pigs has not been particularly successful over the years.

Again, this is some of Diane's work that she already discussed. The response to F1 is more variable than that seen in the mouse. The response to F1 was slower than that to V, which is the opposite of what you see in the mouse. Guinea pig sera can passively protect mice, and there was some protection of guinea pigs observed, but not the level of protection that we would see with the mouse.

There was some evidence of bubo development in immunized animals and very long-term infections here, not a good model of acute disease, and again the response to F1-negative strains is different than it is in other animal models.

Live attenuated vaccines were extensively explored in guinea pigs. Again, like the mouse that you would expect, the results depended on the nature of the attenuation, but there were some

cases where excellent protection was demonstrated.

Some vaccine strains--this is a very interesting paper by Meyer--showed that strains that were essentially avirulent in guinea pigs killed nonhuman primates. The exact title of the paper I think was Strains Harmless to Guinea Pigs and Highly Virulent in Primates, which is a scary sentence in terms of looking at the guinea pig model.

So, here are some statements made over the years about the guinea pig. "The guinea pig is not a suitable animal for testing plague antiserum."

"In experimental plague immunization, the reaction of the guinea pig has been unique. It is not quite like any other animal model."

Finally, "The response of guinea pigs did not offer any improvement over mice in evaluating the efficacy of plague vaccines."

So, these reasons, along with some others that I have mentioned, really have led many investigators away from the guinea pig as an animal model.

A model that has not been as extensively explored is that of the rat. Chen and Meyer, along with Williams and Cavanaugh, and some other researchers in the seventies, did do some research with various types of rats, different species, what they just call the "Sprague-Dawley laboratory rats," I am no exactly sure which one that is, and they found that the subq lethal dose was significantly higher than that of mice or guinea pigs, and that they found resistance to infection, not just in animals trapped in endemic areas, but also animals taken from areas which were not endemic and laboratory rats themselves, so this didn't appear to be acquired immunity.

As a side line, it is interesting to note that they found antibody and resistance to infection was transferred to the progeny of immunized animals.

Based on these results, they divided rats into three groups which they believed to be genetically different. The first are susceptible rats which die from a fairly small dose

consistently.

The second, partially resistant, that is, they survive a small dose. Some of them seroconverted and acquire immunity to a larger dose. Those which do not seroconvert remain susceptible to the higher dose.

What they call resistant, which survive, do not seroconvert, and they remain resistant to even higher doses. There is some evidence that this may indeed be genetic, and, in fact, they derive their strength from the Wistar rat, which they call "WR," and I am assuming it stands for Walter Reed since the researchers were from there. That was highly susceptible to plague regardless of the age of the rat, the sex of the rat, or the season of the year.

So, apparently the seasonal issue was one for rats, as well. Subcutaneous could be, you could use either subcutaneous or an intranasal challenge. They did point out, Williams and Cavanaugh, that intranasal was not as reliable as aerosol, that it led in the rat model to involve

larynx and the tonsils, but they believed it to be a more stringent test of vaccines than subcutaneous challenge.

It has been very rare to find documentation of direct comparisons between animals using the same strain, the same exposure conditions, et cetera, but this is one of them. This is Meyer, Quan, and Larson 1947 looking at intranasally induced pneumonic plague in mice, guinea pigs, and cotton rats.

They did find that they got primary pneumonia in all three models. The mice and the rats had a progressive disease that eventually led to death between 72 and 96 hours. Guinea pigs, however, were not visibly ill until they suddenly dropped dead between 72 and 96 hours, which also doesn't mimic the human condition.

The infection in the guinea pigs in this case was confined to the respiratory tract, whereas, it was more disseminated in the rat and mice.

A model that has not been excessively

explored, but which is interesting, are two species of the multimammate mouse. One of them is inherently more sensitive to Yersinia pestis than the other, M. coucha being more sensitive. There were laboratory colonies established of these models at one time.

It was put forth that perhaps this species might more closely mimic the susceptibility of the nonhuman primate to attenuated live vaccine strains than did the mouse or the guinea pig. This was based on one very small study looking at the strain where the guinea pig was not effective, and these monkeys were dying.

This model also reacted, it was very sensitive to this strain in the same way as a nonhuman primate, so they presented this as an alternative to the guinea pig in that it looked more like a nonhuman primate model in terms of attenuated strains.

It is thought or at least proposed that the difference between these two species of multimammate mouse is that the more resistant

species react to antigens of Yersinia pestis nonspecifically, so there may be an innate resistance there that protects the animal.

There have been Vole models used, also bred in the laboratory, and there has been a small amount of genetic work on this looking at the nature of resistance and there may be an association although it has not been definitively shown between phagocytic activity and resistance at challenge.

One thing that we haven't really discussed, but that should be kept in mind when choosing an animal model is that there is some host specificity of Yersinia pestis. As I have already mentioned several times, F1-negative strains are virulent in mice and in nonhuman primates, but significantly attenuated in the guinea pig.

Certain auxotrophs are also more attenuated in the guinea pig than in the mouse. These include aro mutants, purine auxotrophs, and asparagine auxotrophs, and the asparagine connection has been explored and this is actually

quite interesting. Lynn Boroughs did this.

Guinea pigs have an asparaginase in their sera, which degrades asparagine. So, in that animal model, there is no asparagine available for the asparagine auxotrophs, and the organism is unable to grow, and thus is attenuated. Mice do not have asparaginase. So, that is a very elegant study done I believe in 1971 with some major Brazilian strains of Yersinia pestis that were asparagine-negative.

There are some isolates also from the former Soviet Union, which are virulent for a number of models, but not for guinea pigs, and the reason for this has never really been explained. It is my understanding that these strains are not asparagine auxotrophs, so there has to be another explanation.

So, in conclusion, the mouse is the best established and accepted model in my opinion. The guinea pig has numerous drawbacks, some of which I have mentioned and some which have been described by other people.

There were other interesting models, but they haven't been as well developed, and they need further exploring.

Going back to Otten, it doesn't look like we have really gotten very far since 1936. "It appeared that the nature of the experimental animal was by far more essential to the results than the nature of the vaccine use." Some wise words from people in the past.

So, I will take any questions.

[Applause.]

DR. LYONS: Questions?

DR. : Pat, I just wanted to comment that there several different labs have recent experience with intranasal installation, and I know Rick Lyons has done some, we have been doing it, I think Sue Straley has done some--not yet? Okay. And Virginia Miller.

At least three different pestis strains have been used, several different strains of inbred mice, I don't know that anyone has used outbred. They are all finding that, you know, we get LD50s

on the order of 3- to 500 bugs.

DR. WORSHAM: That would be the general dose?

DR. : That is the delivered dose.

DR. WORSHAM: That is the dose that you deliver into the nares.

DR. : Delivering 50 microliters generally, I think. Generally, all of the lobes of the lungs are involved, the mice are bacteremic within 24 hours, and it seems like a pretty good model for pneumonic infection. I don't want people to go away with the impression that you can't get something that looks like pneumonic plague by doing intranasal.

DR. WORSHAM: You can get something that looks like pneumonic plague, but I have not seen the pathology that really describes the resulting disease processes and whether it is confined to primary pneumonic, or whether you also get cervical involvement. Have you seen that?

DR. : We haven't looked

carefully at the pathology with respect to cervical. I don't know if Rick has or not.

DR. LYONS: I think it is a lot like anything. The technique is critical to that, and we don't see it, but that doesn't mean, you know, giving something intranasally is not necessarily that easy, I mean to do it right. I think again, it is just learning the correct procedure and doing it right.

But I wanted to ask, I guess I am a little surprised even with inhalation that sometimes you don't see cervical, because clearly, most of those bugs are going either in the gut or up in the turbinates or someplace. I mean it is not a lung only, you know, there is a lot of bugs around.

So, has that been carefully looked at for inhalation, too, that there is no cervical nodes or anything, or is that just kind of assumed?

DR. WORSHAM: I think that has been looked at in some experiments. Many of these, a lot of this work is very old. It probably was not well quantitated, and I think it is probably a matter of

quantity. You see obvious cervical involvement or the animal dies, because if the primary pneumonic is strong enough, you may not have time to grow out cervical nodes.

DR. LYONS: Right, and I guess I would wonder if--and this is my own--if the cervical was the dominant mode, I would expect the kinetics to be a little different time to death. I mean pretty clearly, at least for subq versus intranasal or inhalation, the kinetics, the time to death is dramatically different, but I don't know about that. If you tried to infect the cervical node, what the kinetics would be. Do you have any clue?

DR. WORSHAM: I think that parallel experiments would be very nice. I think that that would be a nice study to do where you have control animal sets, strain sets, gross methods to the organism, and actually look at that kind of thing, I think that would be very interesting, but intuitively, it seems like there might be some differences between installing a rather large volume in the nares versus a small particle

aerosol, whether that is relevant, to a large degree, looking at vaccine efficacy, is another question altogether.

DR. : I am curious. Why do you think that the LD50 is so much higher for the aerosol? You are reporting about 4,000? It is about more a magnitude higher than what we see with intranasal.

DR. WORSHAM: I think that there are a lot of variables here that make it very difficult to answer that question. Is it the strain involved? Is it the method of growing the strain? Are some of the organisms damaged by the aerosol?

It could be the mouse strain is different. Like I said, it would be really nice to do some studies in parallel where we are looking to try to control some of these variables, because historically, that makes it very hard to look at this work.

DR. LYONS: Is that calculated dose, or is that actually--are the lungs removed?

DR. WORSHAM: Those are calculated dose.

DR. LYONS: That could explain the whole thing, it probably is.

DR. : I would just reiterate that for people that are doing these studies, they ought to do careful histopathology of the upper respiratory tract. It jogged my memory, and maybe somebody else here can remember from USAMRIID, but my recollection was that Kelly Davis had found, in the nalt antigen, very early--this is after aerosol challenge--

DR. WORSHAM: Mice?

DR. : My recollection was in mice, but I could be wrong. It was a long time ago.

DR. WORSHAM: You didn't publish it.

DR. : I know that, but it is something that people will ought to look at in the different models, because my recollection was that it occurred very early, which was a surprise to us.

DR. : I would just put out a cautionary note. When you use large volumes intranasally, we have actually measured this, and

we use 50 microliters, 90 to 95 percent of it ends up in the stomach, so you may be looking at part of the pathogenesis may be GI rather than a respiratory, so if you stay below 15 microliters, you tend to keep it out of the stomach, so it is something to think about as you do experiments.

DR. WORSHAM: And that is relevant because it was shown many years ago, I think, that you can infect animals orally with Yersinia pestis.

DR. LYONS: Thanks, Pat.

The final speaker for this is going to be Louise Pitt from USAMRIID talking on nonhuman primates as a model for pneumonic plague.

Nonhuman Primates as a Model for Pneumonic Plague

Dr. Louise Pitt

DR. PITT: Good afternoon.

Now for the very interesting and complicated topic on nonhuman primates and which model to use for pneumonic plague.

In order to understand and appreciate the models that are used today, I think it is very

important that we go back to the beginning because the work that was done right at the beginning has influenced all our decisionmaking to date.

It started off in the 19th century in Indochina where initially it was discovered by some Russian workers. They inoculated some nonhuman primates with the organism that is for this plague, three species of monkeys, and found that they were very susceptible.

It was only in 1933, though, when Taylor went back and identified these three species that were used - the three macaques, Macaca sinica, radiata, and Semnopithecus or the Presbytis entellus, which is also known as the Langur.

In 1898, then Yersin had what he called an "attenuated" strain. It actually killed rodents, but was termed attenuated. He, being a classical scientist, did what they did in those days, he took some macaques and himself, and he inoculated both. Both himself and the macaques got fairly ill, but survived, and his conclusion was that the susceptibility of the macaque was similar to man.

In 1899, some German scientists then put Yersinia pestis into the Langur and compared it to the Macaca radiata, saying that they were similar in susceptibility.

Again, in 1904, a nonpathogenic strain for guinea pigs was found to be virulent, in this case, in Cercopithecus aethiops, which is the Grivet, which is also the African Green monkey or the Verbit, very similar.

1907 was the first time when a cynomolgus macaque, the philippensis cynomolgus macaque was used, and it was determined that the susceptibility of the cynomolgus macaque lay somewhere between the Langur and the Macaca radiata.

Again in 1912, the cynomolgus macaque was used and shown to be much more susceptible than guinea pig.

Now, in order to put all this historical susceptibilities into perspective, we need to remember that all of these relative susceptibilities of these nonhuman primates was based on inoculation of the skin.

They all concluded that the macaque showed individual variations. This, of course, is very old studies, unknown where the macaque came from. Some were caught from the wild, the majority were actually caught from the wild. Studies were done under fairly primitive conditions. There was lack of technique standardization, cultures varied, very little information as to strain information. So, all this data needs to be taken in that perspective.

However, there was always one conclusion that was agreed upon across all the literature, that regardless of susceptibility, once the animals became ill, the disease was very similar across the different nonhuman primates and was also similar to what was seen in humans.

This is just a summary of the table from Meyer in 1954, where he showed a single--this is 195/P, a virulent strain of Yersinia pestis. As you can see, many experiments with Macaca mulatta, also subq route, but you get survivors from 2 logs to 9 logs, and in cynomolgus macaque, 10

given subq, you still get survivors.

The conclusion was always that the macaque was susceptible to plague Yersinia pestis, but that there was individual variability, considerable individual variability. A group of animals brought in from a single site could have an incredibly different susceptibility to Y. pestis.

Now, moving on to pneumonic plague, the initial study by Ehrenkrantz and Mayer in 1955 looked at Macaca mulatta, the Rhesus macaque. This was actually intratracheal, not an aerosol exposure, and came to the conclusion that about 100 CFU of this 195/P strain killed more than 50 percent of the animals. So, quite a difference from the skin inoculation route.

Speck and Wolochow, in 1957, did some aerosol work. This was small particle aerosol using the Macaca mulatta again, the Rhesus macaque, and concluded that their LD50 was around 2 x 10 4 of the 139L, a virulent Y. pestis strain.

On looking at this again several times, it appeared that there was a vaccine study going on at

the same time and any animal that died was actually included in this LD50 estimation, including animals that had been partially immunized with a vaccine, so any animal on this trial was included in the LD50 study, and this could very well be why the LD50 is so high in these studies.

Moving on now to the early 1990s, at USAMRIID, when we set about developing the nonhuman primate model for pneumonic plague, we did extensive literature research, had extensive discussions, and the species that was chosen was the Cercopithecus aethiops, which is known as Chlorocebus aethiops or the African Green monkey, and as I said, this was based on a very extensive literature review, because throughout the literature, this model was the one that was consistently consistent. That was the message across all the studies, that it was a very, very reliable consistent model.

We realized based on susceptibility that it was susceptible, probably more susceptible than the macaque, but we chose it because we were

looking for a stringent model, and then based on our literature survey and then the work that I am going to show you now, the course of disease in this animal model is very similar to vaccine in humans, and death is due to primary pneumonia.

This is the analysis of the LD50 curves for the African Green monkeys, the strain of Y. pestis that was used in Colorado 92. This is the standard strain that we have used at USAMRIID for all our challenges, whether they be for vaccine efficacy or therapeutic.

We have also used the F1-negative isogenic strain of Colorado 92, which is called C12, and the LD50 is around 343 CFU, and that is an inhaled or presented dose, so this is a very susceptible organism.

The LD99 based on this curve is around 50 LD50. We did do an LD50 study with the F1-negative strain and got an LD50 of 800 CFU, very similar.

Based on a study that was done not too long ago, this is the clinical pathway after an animal has been exposed. This is a natural history

study that was done in conjunction with a therapeutic study, but it shows the model very nicely.

The animals are exposed at time zero. They have telemetry devices in them, so we monitor their temperature all the time, and you can see they are perfectly normal 24, 48 hours. Around 72 hours, the temperature increases, they have a fever until they succumb and die. Usually, we euthanize the animals whenever possible when they become moribund.

This is an example of an animal that received 57 LD50. This temperature curve is very consistent, this model is very consistent. At the time when they are getting a fever, there are no clinical signs, but within 12 to 18 hours, they do start to show clinical signs. At the time that they show clinical signs, they are bacteremic, and then death occurs fairly rapidly after that.

This is the heart rate curve showing a very similar pattern to the fever curve.

This is showing you the respiratory rate

that is fairly steady until you get towards the last 12 hours or so prior to death where you get a massive increase in the respiratory rate.

This is the pattern of the white blood cells showing around 72 hours you get that inversion of the lymphocytes, granulocytes with the monocytes staying fairly constant.

These are radiographs of the same animal. This was taken pre-exposure. This radiograph was taken at 83 hours, at a time when the animals were showing clinical signs. You can see there is some infiltrates in the lung at this time, and at this time, the animal is bacteremic.

The last radiograph was taken when the animal was euthanized at 111.5 hours post-exposure.

This is just another view of those radiographs, before, during. The very rapid time course of this disease.

More recently, after many discussions with collaborators, et cetera, we went ahead and we developed the cynomolgus macaque as a comparison model. We did an LD50 based on the staircase

method, ranging in the 10

4 to 102 CFU range, and the

LD50 came out to 400 CFU Colorado 92.

It turns out that the cynomolgus macaques are just as susceptible as the African Green monkey based on the data and the consistency of the experimental conditions. They die within the same time range, 4 to 5 days.

The onset of fever in animals receiving a lethal dose is similar, around 72 hours post-challenge, and the animals are normally moribund within 48 hours post-challenge.

Looking at the clinical signs between both African Green monkey and the cynomolgus macaque, no real difference whatsoever. Fever is the initial symptom. There is an increased respiratory rate. These animals then breathe extremely rapidly with labored breathing and rales.

They are usually euthanized when they are moribund, and at the time of euthanasia, pink froth just pours out of their mouth and nose.

This is just some of the comparative pathology that is being collected to date. We

don't have as many macaques as we do African Greens at this point.

This table just compares the control African Greens to vaccinates that have died, and smaller numbers of untreated macaques, and we have, in this table, only two vaccinated macaques that have died.

In the African Green, the symptoms are very similar in the vaccinate versus untreated, and the untreated African Green and the untreated macaques have very similar pathology.

Now for some pathology. I am not a pathologist, but I have to show you some pathology.

This top one is the normal lung. This is a vaccinate, exposed lung from an animal that is a vaccinate that died, and this is the control lung. This is actually from a cynomolgus macaque. If the slide was from an African Green monkey, it would look exactly the same.

Here, you can see there are neutrophilic infiltrates in the lung. This is pathology of the spleen, the red is spleen. The blue in here is

bacteria, and again you can see that there are neutrophilic infiltrates.

Here, there is liver, and this is a fibrin thrombi that is covered with bacteria.

So, in comparing the African Green monkey and the cynomolgus macaque to date, based on clinical signs, the disease progression, and the pathology, as well as susceptibility in terms of an LD50, they are very similar, very similar in susceptibility, pathology, and disease progression, and both are very similar to what is known about the human disease.

So, now moving on to vaccine efficacy, first of all, in the African Green monkey, the initial study that was done at USAMRIID back in the early 1990s was looking at the plague USP, the licensed vaccine. You will notice the Cutter vaccine.

We had 12 vaccinates and 6 controls. They were given the licensed schedule, at day zero, 28, and 91, and then the animals were challenged about 7 weeks later. The challenge dose was around 118

LD50, and there were no survivors, and there was no difference between mean time STAT.

At that time, we measured antibody responses to F1, and I did not bring the data, but at time of challenge, the F1, anti-F1 titers were very low. With this vaccine, the IgG titer would go up after every boost, but then come down fairly rapidly.

Now, moving on to the more modern times, efficacy of the candidate recombinant F1-V fusion protein vaccine that was developed at USAMRIID. The study design, and this is a competent study design because I am going to show you several studies.

Basically, the vaccination route was always intramuscular. The F1-V fusion protein was always combined with alhydrogel. There were either 2 or 3 doses given. The challenge was always 6 weeks after the last dose, and, of course, the challenge was always the small particle aerosol, and we used either the Colorado 92 or the F1-negative strain.

Some results. The first study, the animals received 2 doses of 30 micrograms of the F1-V at zero and 28. The challenge strain in this study was V12 with an average of 55 LD50 as the challenge dose, and 2 out of 4 survived, whereas, the one control succumbed to pneumonic plague.

In the next study, the next group of animals received 2 doses of the 30 at zero and 28, and then at 3 months, received a 300 microgram dose of F1-V to see if we could boost the survival. They were again challenged with the V12 strain with a higher average LD50 of 259, and 3 out of 4 animals survived while the control succumbed.

In the next study, animals received 150 micrograms of F1-V 3 times, at zero, 28, and 56. The challenge dose in this study was much higher, at around 600 LD50, and 4 out of 10 survived while the control died.

The next study, again F1-V, 150 micrograms, 3 doses, the same schedule. This time they were challenged with Colorado 92 with the average dose of 166 LD50, with 2 out of 10

surviving.

The final study, again, the exact same study design, 150 3 times. Challenged with Colorado 92 with a lower challenge, but zero out of 10 survived in that trial.

This is the immune response data from the last study that was shown, showing that the animals got antibody to F1, a fairly consistent response. All the animals responded to F1 and had IgG. The antibodies to V in the African Green, it is a very varied response, and this is pretty much typical of all studies that have been done, that the response to V antigen in the African Green is a very individual and varied response.

Moving on to vaccine efficacy now in the cynomolgus macaque. First of all, the recombinant F1-V fusion, the USAMRIID candidate. The study design, basically, very much the same as the African Green monkey study with 3 doses given intramuscularly, challenged 6 weeks after the last dose.

This is the immune response data for both

F1 and V, and in the cynomolgus macaque, the V response is much more consistent.

Results. Three trials to date. The first, 150 micrograms given 3 times, zero, 28, and 56 days, just like with the African Green. The challenge strain Colorado 92. The average challenge 72 LD50 with 80 percent survival, 8 out of 10.

The second study. Again, exactly the same study design. In this, a very low LD50 challenge was given. All the animals survived and both controls died.

Again, the third trial with 160 LD50 average, challenged, 8 out of 10 survived.

Now moving on to the other recombinant F1 and recombinant V protein vaccine. This is the vaccine developed at DSTL in the UK, and this was a collaborative study between the DSTL and USAMRIID with funding from the Joint Vaccine Acquisition Program here at DoD.

The study design, the vaccine antigens are the recombinant F1 and the recombinant V protein

that are combined with 20 percent volume to volume alhydrogel. The vaccination route is intramuscular. Two doses were given, and as standard with all the vaccine trials, the challenge was 6 weeks after the last dose.

This is the immune response data for both the F1 and the V antigen, again showing the consistency of the response of the cynomolgus macaques, both F1 and V.

That first one was for the 40 microgram. I forgot to mention there were 2 doses of this vaccine given. The one group got 40 micrograms of F1 and 40 micrograms of V, and in the second group, it was 80 micrograms of F1 and 80 micrograms of V, and this is the immune response to the second group that got 80 plus 80.

The results. The group that got 40 plus 40, the schedule was zero and 21 days, the 2-dose schedule. The average challenge for this study was 126 LD50. We did lose one animal prior to challenge to an unrelated event, and 8 out of 9 animals survived with the 40 plus 40 dose.

In the 80 microgram plus 80 microgram, 10 out of 10 survived, and the 2 controls died.

So, to summarize to date, it appears based on the disease process and the clinical signs and what is known of the human disease, both African Green monkey and the cynomolgus macaques are appropriate models for pneumonic plague, and recombinant F1 and V-based vaccines do provide protection against the lethal aerosol challenge, and as I said previously when we started, we chose the African Green because we felt that they would be a more stringent model, and I think the results to date have proven that they really are a very stringent model in terms of vaccine efficacy and a very high bar.

Time will tell which is the most appropriate model.

Thank you.

[Applause.]

DR. LYONS: We have time for questions. Louise, I just have one quick question. I don't know if you have any information, but is the

poor immune response, do you know if that is just to V antigen, or is that characteristic of green monkeys for any antigen that you are aware of?

DR. PITT: The F1 was characteristic. It is pretty much the same. We are in the process of looking at other antigens in African Greens to see what that looks like, but in terms of this, in all the literature, there isn't anything to point the way, no.

DR. : This might be a very naive question. Given the wide variation of LD50, you see different study, even within sometimes the same group, and different challenge and strain. From previous, other presentations, people use index.

Is that possible to use something like that as a more quantitative or also included variable how long the animal will die instead of just LD50?

DR. PITT: In our hands, the LD50 is very low and very consistent both for the African Green and the cynomolgus, and that is with an aerosol model of pneumonic plague.

I think the variation in the species and susceptibility comes when you look at the skin inoculation, and I think you might see, if you do bubonic studies, that there might be quite different susceptibilities between these two species in terms of an LD50.

DR. BURNS: Drusilla Burns, CBER.

Louise, in your vaccination studies, your African Green monkeys weren't protected very well with the fusion protein. Do you think that was simply because of the very varied response that they had to the V antigen? Did animals that died have very low response to V, or was there no correlation?

DR. PITT: In terms of the small number of animals that we got to date, and the ELISA data, there doesn't appear to be any correlation other than some animals appeared to respond to V as if they have seen V before, and others appear to be more naive to V, and I think there needs to be a lot more work done on that subject to understand exactly what is going on.

I didn't have time to put up a lot of detail on different experiments, but if you look at the studies of the animals that survived in the African Green versus those that died, there is a trend towards the lower challenge dose, the more survive. So, that would lead us to that this is a much higher bar to reach in terms of the model.

DR. BURNS: Do you have any idea where humans are on this scale?

DR. PITT: I would not like to comment.

DR. : Have you established any sort of a target LD50 in selecting the LD50s that you use in the sense that what sort of level of exposure might be expected during a bioterrorism attack, for example, what level of protection do you need?

DR. PITT: That's a good question. There could be many answers, because it would depend on the scenario in which you would be exposed. Given that the LD50 is so low in nonhuman primates, and assuming that it is also low in humans, you wouldn't have to be exposed to very much for it to

be a lethal dose.

MS. SCOTT: Leah Scott, DSTL.

I was just wondering whether some of the variability issues that you saw with the African Greens might be explained in part by some of their sourcing issues and their rather different natural history that they would have been exposed to perhaps in early life.

Perhaps as a follow-on to that, would you see that as a potential problem area when one considers a requirement to do key studies to GLP in view of like breeding programs?

DR. PITT: Well, the African Green monkeys, actually, I think why such a consistent model is because all the animals we have ever received have come from the Island of St. Kitt, which is basically a closed colony, has been for over 300 years, so the source of the animals has actually been exactly the same.

MS. SCOTT: But they would have been exposed to a much wider range of natural stimuli presumably than most captive bred animals.

DR. PITT: Possibly.

MS. SCOTT: As I was saying to you earlier on, we are proposing to complicate the issue yet further by proposing, as we are trying to do in the UK, to look at the common marmoset as a potential model in these areas, and we have already characterized the species, so it would be very interesting to put all of these results together and compare.

DR. PITT: It certainly would, but I would also add that cynomolgus macaques are not captive bred. They are also brought in from outside sources, so they have also been exposed to external stimuli.

DR. MEYSICK: Karen Meysick FDA.

I was wondering if you would comment on the differences between cynos and the African Greens in terms of just their background. My understanding was that African Greens, I think are more susceptible or resistant actually to SIV.

DR. PITT: Right, they don't die from SIV, correct. They can be infected, but they don't

actually develop the disease.

DR. MEYSICK: As I related to, it is something to do with CD4s, I think, CD4 cells.

DR. PITT: Right. The TB ratio in African Greens is reversed compared to macaque, but on that subject, I think that is a very important discussion point when you are talking about animal models, how do we actually know about the animals themselves, let alone adding in organisms to make them sick.

DR. : I should know this, but were the serologies done by the same assay or the same group between the F1-V fusion and the F1 plus V?

DR. PITT: No, they were not.

DR. : It would be useful to do that.

DR. PITT: I think it would be a great exercise.

DR. : Because clearly, differences in the level of antibody, and that would be important to see if there are functional

differences.

The other point is in all of these studies, that somebody needs to consider what, if anything, is F1 doing.

DR. PITT: Yes.

DR. FERRIERI: Pat Ferrieri, University of Minnesota.

I have seen LD50s in the literature cited as 3,000 for humans. Do you have any notion where that would have been derived from, any data that you have seen anywhere?

DR. PITT: No, I think that is based on assumptions of what people know from the Manchurian outbreak, from information that has been received from different documents, but I have no idea where that number comes from.

DR. FERRIERI: Another quick question. I also have been concerned about the genetic lineages of these two types of nonhuman primates, and do you have multiple sources from which you obtain your animals, breeding facilities are different or not?

DR. PITT: As I said, the African Green

monkey, we have been very consistent with the source of the African Green monkey. The cynomolgus macaque has been a little different because they have been come in from the Seychelles, they have come in from the Philippines. They have come in probably from China, too, and India, I believe. So, there could certainly be differences in the cynomolgus macaque that we are not aware of, but the African Green source has been consistent since we started working on them.

DR. LYONS: Is the V antigen in the fusion, is that functional? Like if you put the fusion protein on cells, do you get the IL-10 response and that sort of thing, do you know?

DR. PITT: That is not my area of expertise. I believe it is functional.

DR. LYONS: I am just curious because it looked like on your slide, that the 30, you know, and this was small animals and everything, that the 30 tended to work better than the 150 dose, at least for CL12, and the question would be could you go up high enough with proteins, so that you now

you do bring out this local immunosuppressant phenotype, and actually by giving too much protein, you decrease your immune response.

I find that hard to believe.

DR. PITT: We have had those discussions.

[Recess.]

Session 4: New Data on Aspects of Plague Vaccine Development

Dr. Luther Lindler, Moderator

DR. MEYSICK: I think we will start the last session for today, which is new data on aspects of plague vaccine development, and the moderator for this session is Dr. Luther Lindler from the Department of Homeland Security.

DR. LINDLER: Thanks. This session is on new data on aspects of plague vaccine development, and the first speaker is going to be Sue Straley speaking to us about how does antibody against LcrV protect against plague.

How Does Antibody Against LcrV Protect Against Plague?

Dr. Susan Straley

DR. STRALEY: We are trying to discriminate among the various ways that anti-V could possibly protect against plague, and I would like to tell you about one of our stories.

Our model is Yersinia pestis KIM delta pgm, which is essentially fully virulent from an intravenous route, so our inspection is going to be intravenous, and it will model systemic plague. We have a very potent rabbit polyclonal antibody antiserum against LcrV that we give to the mice in one dose the day before we infect, and we give a high dose of bacteria to allow us to follow the dynamics of early protection by viable numbers.

So, in our control mice, which are C57 Black 6 mice, we look at the gold and the blue symbols, if the mice are given the anti-V shots and they control bacterial numbers, these mice will live. If they were given instead a nonprotective anti-Yop and antibody, then, they experience a runaway infection and will die starting around day 4.

So, the first question we wanted to ask

was can you get protection in the absence of V's effect on IL-10?

We can address this in two ways. On the left we use IL-10 knockout mice, and these are highly polarized toward Th1 responses and are actually remarkably resistant to plague lethality, but nonetheless, you can give a high enough dose and kill them, so we tested, and the answer was yes, we got exactly the same dynamics, these mice are protectable by the antibody, and we did it a different way over here.

I am showing just one time point where we ablated IL-10 with a neutralizing antibody against it, and the controls with anti-YopM received neutralizing anti-IL-4.

So, the answer is yes, you can protect using anti-V in the absence of effects relating to IL-10 production from V, and so the issue was then, what is it. One thing that we followed then is the effect on Yops delivery, and we have confirmed in a number of assays the findings of the Welkos group and others that anti-V does partially prevent the

delivery of Yops to adjacent cells.

In this context, it is useful to look at this particular assay, which is a phagocytosis assay using a double fluorescence method. So, if the infection is done in the presence of the nonprotective antibody, the anti-YopM, then, most of the bacteria are extracellular and stain red, and if it is done in the presence of the protective anti-V antibody, then, most of the bacteria are intracellular.

So, that raised a question, does the bacterial location affect the expression and delivery of Yop?

There was an experiment that Roland Rosqvist and Hans Noskos did back in 1990 with ED76 in HeLa cells that indicated that the only bacteria producing Yops were the extracellular ones, and we wanted to know is this the case for Yersinia pestis KIM and J774 cells.

So, here is the design. We are going to make a delivery of YopH into the cytocellular fraction of J774 cells after four hours of

infection. We have set it up this way. We have a whole bunch of cultures. Some of them are infected in the presence of the anti-V antibody, some with anti-YopM, some with no antibody, and this goes for 30 minutes to allow phagocytosis to take place.

There will also be an initial burst of Yops delivery during this time, and then triplicate cultures are divided as follows. So, one gets a dose of gentamicin which is sufficient to inhibit protein synthesis by extracelluar Yersiniae, so that now the only further Yops that are going to be delivered would be by intracellular bacteria.

One gets a mixture of antibiotics that will kill all Yersiniae inside and outside in 15 minutes, so they will be essentially no further Yops delivery, and one gets no addition. So, here, we are going to get Yops from both extracellular and intracellular, and this goes for an hour. That is washed away, and the antibody treatment is restored, and then the incubation is finished.

So, here is what we got. The no antibody, anti-YopM, anti-V, and the three drug treatments

for each, this is noninfected. I would like you to focus on the comparison of gentamicin and mix in each case.

The mix kills everything. You get no further Yops. Gentamicin, you get delivery from intracellular bacteria. They are pretty much the same. There is no delivery from these intracellular Yersiniae, and what is happening here is what happened during that first half an hour.

If there was no drug treatment, then, you got tons of Yops still delivered, presumably by the extracellular bacteria in these control treatments. With anti-V, when the bacteria are mostly intracellular, you obviously get less Yops delivered.

So, that raises the question: Is antibody inhibiting Yops delivery and causing phagocytosis, or is antibody causing phagocytosis and subsequently inhibiting Yops delivery?

So, to test that, we asked can antibody be effective in preventing delivery of Yops to cells that cannot phagocytose.

So, we did these infections in the presence of various concentrations of cytochalasin D, and when there was no cytochalasin D, then, yes, you do get some inhibition of Yops delivery by anti-V antibody compared to the controls, but as we increase the cytochalasin D, we got progressively less and less effect of the antibody.

So, antibody is not effective against bacteria, against cells that cannot phagocytose, and that leads to the question of what is it about antibody, is the ST portion of the antibody important for its efficacy, and the answer is yes. This is actually in full agreement with an experiment published by the Welkos group. So, with no antibody, most of the bacteria are extracellular. With full length V antibody, you get promotion of phagocytosis, but if you make FABs, it doesn't work very well.

So, this is what we think is happening, that antibody is actually promoting phagocytosis and consequently, you get inhibition of Yops delivery rather than the other way around.

So, if phagocytosis is so important, and we think it is important to protection, then, what are the cells that are important or mediating this. So, we evaluated the relative contributions of macrophages and PMNs.

To do macrophages, we took advantage of a recently available transgenic mouse model that allows you to conditionally ablate cells of the macrophage lineage, and so we are seeing here then our usual control mice of the nonablated that receive anti-V or anti-YopM, but the ablated mice that receive the anti-V, it really made no difference in spleen.

So, the macrophages are either redundant or not necessary for protection in spleen. In liver, we got a small effect, maybe about 10-fold less ability to contain bacterial numbers, so macrophages do make a contribution in liver.

We ablated PMNs with an antibody against ra-1, and in that case, it made a huge difference in both organs. You get total loss of ability to contain bacterial numbers.

So, I have shown you that, as we all know, and I really believe, anti-V promotes phagocytosis and that this is actually crucial for its protective effect; that Yops are not delivered by phagocytosed Yersinia pestis. Yops are crucial for growth in organs. I didn't show you those data, but they are, and that is what anti-V is doing. It is preventing growth and thereby you never get the bacterial numbers to produce enough V to even have a big effect.

I told you something about mediators of early protection, that PMNs are really important, and macrophages also make a contribution.

This work was almost entirely done by a postdoc Sasha Philipovsky, some help from Clarissa Cowan, an advanced technician, and another technician, Michael Gray.

I will be happy to answer any questions.

[Applause.]

DR. : Very nice, Sue. This is just a comment. You think it goes to say that an earlier study by Friedlander and colleagues showed

that the immunization of mice with another protein which is thought to aid in the translocation process, namely, YopD, doesn't even nearly promote the same protective efficacy as antibodies against V.

That will corroborate the notion that what the V antibodies may be doing is not blocking the injection of YopD.

DR. STRALEY: We haven't evaluated what antibody against D does, YopD does. I really can't address that relative efficacy.

DR. : What the data show is that the antibodies against D do not necessarily block the injection of Yops from extracellular bacteria.

DR. STRALEY: If the bacteria remain extracellular, they deliver Yops, I think is the fair way to say it, but somehow antibody against V is also promoting phagocytosis, and I think that that is what is causing the downregulation of Yops that we see.

DR. : I am curious if you are not far enough to find out if acidification of the

vesicle is necessary to prevent infection, because it would seem that infection could conceivably occur through the vesicle itself.

DR. STRALEY: We have not tested that. DR. : Do you think that the effect of anti-V is specific to the FC receptor in that it is that mechanism of phagocytosis that is critical, or do you think if you could promote rapid phagocytosis by any mechanism, you would get the same response?

DR. STRALEY: Well, let's see. I guess I can draw on the published literature first, that the Welkos group did show, and Sue may have shown this slide, that you just use an anti-Yersinia antibody, it can protect.

I am thinking of promoting phagocytosis. I guess she didn't show the phagocytosis data today. We don't really know the answer to that at this point. We did test whether we could protect in FC-gamma 3 knockout mice, and you can, but mouse PMNs don't have FC-gamma 3, so I am not sure it was a good test. We are actually doing that right now

to try to address that very question.

DR. : I guess I am wondering also if it's possible that the antibody, if it's binding to the tip of the type III secretion system, it could be essentially preventing the type III secretion system from contacting the host cell at the same time that it's promoting uptakes.

Do your experiments address that possibility?

DR. STRALEY: Well, I guess in the experiment where the cells could not phagocytose, we had lots of antibody around, and we got tons of Yops delivered, so the antibody is not preventing delivery directly.

We have done one experiment also with FAB primes in vivo to see if it would protect, and the answer was no. We are repeating that now. I don't know if anybody else has done that experiment.

DR. : One other question. Do you know the protective epitopes of this antibody preparation you are using, have you mapped what regions of D are recognized by this antibody?

DR. STRALEY: I imagine it's the whole protein. Interestingly, FABs of this antibody will reduce the IL-10 effect, so I am suspecting that a range of epitopes are represented.

DR. LINDLER: Leah Scott from DSTL is going to speak about the marmoset as an immunological model for plague, just one added five-minute talk.

The next speaker is Dr. Stephen Smiley from the Trudeau Institute to speak about cell-mediated protection against Yersinia infection.

Cell-mediated Protection Against Yersinia Infection

Dr. Stephen Smiley

DR. SMILEY: Thank you. I thank the organizers for inviting me. I am a relative newcomer to this field and I am looking forward to speaking to you today.

So, basically, we are asking this question, can vaccine-primed CD4 T cells protect against pneumonic plague, and I am not going to answer that question today, but I am going to show you the tools that we are developing that we think

will let us address that question.

We have already heard today that Yersinia can be intracellular bacteria and that it has been established that interferon-gamma and TNF-alpha, which are products of cellular immunity, can protect against Yersinia infection, and it turns out that it is well established that CD4 T cells are important players in both cellular and humoral immunity, but the actual functional roles during Y. pestis vaccines, I don't think it has been evaluated decisively. It has been shown that they can be stimulated but not shown that they can protect.

So, V protein has already been discussed today in some detail, as has the fact that the vaccine that is under development by USAMRIID of the F1-V fusion protein fails to fully protect primates. What I was told about that was that in some animals, there was a late breakthrough of disease, and what that suggested to me is one possibility is that perhaps there are reservoirs of bacteria that antibodies were unable to clear and

that subsequently, it led to disease.

So, obviously, that is just a hypothesis and so is this, which is that appropriately primed CD4 T cells may be able to direct cellular immunity at those intracellular reservoirs, thereby improving plague vaccine efficacy.

I just want to stress at the outset, though, that our intention is, our belief is that these cell-mediated protection will synergize with the humoral immunity. I am not suggesting that we replace antibodies.

Our approach to this is relatively simple. At the outset, we are going to define CD4 T cell epitopes in V protein. That will give us tools, a way to specifically prime CD4 T cells. Our plan was to assess the protective capacity of those cells, as I said, to assess whether a cellular and humoral immunity can synergize in combating plague.

This is just a schematic of V protein, and these are the peptides that we had synthesized in 96 well format that spanned V protein, and we then screened these peptides in an ex vivo assay.

To do that, we got the F1-V fusion protein from Jeff Adamovicz, from USAMRIID, and we vaccinated E6 mice with that protein to get a strong Th1 type response. We then purified those CD4 cells from those mice after six days, and in vitro did a re-stimulation assay looking at responses to these peptides.

What we were able to clearly see as we scan across this slide, there are 63 individual peptides that were screened. In the top are the CD4 cells from the F1-V primed mice, and in the lower panel are CD4 cells isolated from OVA-albumin-primed mice as control, and you can see that there were regions of V peptides, V1, V2, and V3, where we saw strong responses.

On the far right you can see the controls, the OVA responded to OVA only, and the F1-V in culture only revoked a response from F1-V primed cells.

We went on to further define these epitopes by making a second set of peptides in which we truncated at either the amino or carboxy

terminus by 2, 4, 6 amino acids, and then rescreened in the same type of assay, and that allowed us to find map with specific epitopes.

Those are shown here on this slide, which compares the V proteins from Y. pestis, enterocolitica, and pseudotuberculosis. The peptides that we use is V1, V2, and V3 for vaccination studies on bold, and the boxed are the better defined epitopes by that second series of studies.

You can see that they are completely conserved among Y. pestis, enterocolitica, and pseudotuberculosis. I suspect that it is just coincidence, but it is a useful coincidence for people studying those infections, as well.

It is also useful to us, and I will show you that in a second.

If these are true CD4 epitopes, then, one should be able to vaccinate mice with these small peptides V1, V2, and V3, and elicit responses to those peptides, and that is what this slide shows.

Here, we have vaccinated either with a

control negative peptide V neg, V1, V1, or V3, again in CFA, and then in this particular experiment, we vaccinated on day zero. We then vaccinated with the same peptides in IFA at day 30, and this is looking at a recall response in vitro. It's an Ellis spot response where you can measure the number of interferon-gamma producing cells in that culture.

You can see that the V1 evoked a response from V1 in culture, V2 from V2, and V3 from V3, so these epitopes prime antigen specific CD4 T cell responses.

We then wanted to ask whether these could protect, and in the enterocolitica system it has been shown that cellular immunity by CD4 T cells can be protective, and also enterocolitica is an agent that we could work with easily at that time, so we went there.

Here, I am showing protection. There was significant protection. There are the exact same animals that I just showed, from the same cohort of animals that I just showed you that Ellis spot

data. You can see that the mice that were vaccinated with V2 showed significant prolongation of survival upon infection with 104 of enterocolitica IP.

So, our plan, of course, is to go on and test this with Y. pestis. We set up a collaboration with David Perlin at PHRI who is setting up the intranasal model, but before that got underway, the pgm-minus strains were released from the select agent list, and we were able to test those ourselves, so we got one KIM D27 from Robert Brubaker, and in the exact same type of scenario I just showed you on the previous slide, we were unable to see protection using that strain.

We have tried that several times, and so far that type of protocol has failed to protect against IP KIM D27. I know IP is not the preferred route that people are studying, and we are looking at other routes at the present time.

I just wanted to point out I think as some people have today, that these pgm-minus strains are attenuated, but they are conditionally attenuated,

so by the IP route, this is just an LD50 curve, and you can see that the LD50 is quite low, so they are not so attenuated by the IP route, likewise by the IM route, they are not particularly attenuated. So, we plan to use this for our future vaccination studies.

So, what we are now focusing on is whether CD4 T cells can synergize with humoral immunity to protect. As I said, we didn't expect them necessarily to protect on their own.

In the types of studies that have classically been done along these lines, one can transfer and give serotherapy the day before doing an infection, and then give the infection the next day and measure the capacity of that serum to protect.

But in our types of studies, that wouldn't be appropriate because in these types of studies, what happens is the serum unblocks infection, and we want to ask whether T cells can contribute to clearing intracellular bacteria, so we need to have cells infected first.

So, we decided to try this other protocol where we would first infect and ask whether serum can protect the next day, after the infection is in place.

The answer is--I am not sure whether this has been done or not in the past--but it clearly can. That is the block there, the round circles.

That is serum given day plus 1, again with this 10 4 IP KIM D27 model.

So, post-exposure serum therapy can protect, and it is actually extremely potent. We have been quite impressed. As little as 3 microliters of this serum protects. I forgot to tell you what this serum is, I apologize. When we do a sub-lethal KIM D27 infection, so that is 10 2 IP, we can then collect the serum from those animals out 30 days, and that is the serum that we are using in these serotherapy experiments.

So, that serum is extremely effective, and it has allowed us to do the last experiment that I want to show you, and that is to develop an assay for protective cellular immunity.

So, here you will see I have switched to MUMT mice. MUMT mice lack V cells, they can't make antibody responses. They are genetically deficient in D cell production. So, what we have found we can do in these mice is we can give them the KIM D27 IP infection, and then the next day give them this serotherapy, and if one does this that, these mice survive.

What we wanted to know was did that process vaccinate them with cellular immunity that could protect against a subsequent day 50 challenge. An appropriate control was to use mice that got a sham infection initially, got the serotherapy, so if there is any leftover serum from the lower infection, it should still be there from the upper mock control, as well, and then challenge those mice.

We need to repeat this, but in the first experiment, it was really quite striking. It appeared that all of the mice that were vaccinated, all the MUMT mice which can't make antibodies were vaccinated in a way that allowed them to survive,

were able to then survive a secondary infection with 10 4 IP KIM D27.

So, we are quite excited about this. I think it is pretty clear evidence that cellular immunity can protect against IP plague, and we are moving on to look at the other models.

In closing, I just wanted to bring out a point that I don't think has been discussed much, but for cellular immunity, I think we need to rethink what are the right targets.

V protein is clearly a good target for humoral immunity, but for cellular immunity, in order for cells to attack infected cells, we need the Yersinia proteins to be expressed within those infected cells, and I am not sure V protein is the right target in that context.

We have got assays that we are setting up to try to identify what are the right targets in vivo, and I would be happy to talk to people about that, but I don't think I have time right now.

So, in closing, I just want to thank Michelle Parent and Kiera Berggren who have done

most of this work, my Trudeau Institute colleagues, Jeff Adamovicz for providing the F1-V and support, and Bob Brubaker for providing the KIM D27.

Thank you.

[Applause.]

DR. : Very interesting presentation. I just wondered whether you thought that the T cells that you have identified in the C57 Black 6 mice might be conserved in other haplotypes of mice or not.

DR. SMILEY: I suspect that they won't be. I think that is one of the difficulties of looking at cellular immunity, that since it is all MHC restricted, it will depend on which strain you are looking at. I hope others are looking at other strains.

DR. LINDLER: Any other questions?

Thank you.

The next speaker is Shan Lu from the University of Massachusetts. I think he is going to speak about search for an optimization of protective antigens for plague vaccine development.

Search and Optimization of Protective Antigens for Plague Vaccine Development Dr. Shan Lu

DR. LU: First, I would like to thank the organizers for inviting us to present the data here. Also, I think that this is really a well-organized conference. Being a beginner to this new plague vaccine field, I thoroughly enjoyed the informed conference here today.

When we start plague vaccine, actually it was started as a graduate student vacation project. Being someone working in the vaccine field for 10 years including some of the HIV project, I hope people appreciate how much you have here.

Actually, you have an animal model, you have something of the immune correlates, and actually you know what antigen they protect.

So, when we started, we look from different perspective, that is, what is the issue we want to address here, how can we improve rather than reinventing the whole wheel. So, I thought we should divide it into two parts.

One is how can we improve the immunogenicity part and then the second one, especially in the current regulatory and society environment, how can we improve the safety of the future generation of plague vaccine.

In the immunogenicity part, we know that at least the two protective antigen has been identified, however, the quality, especially the production, how do you put the two antigen together has been some issue. When you fuse them, do they really form a big aggregate or a functional antigen?

Also, we realize including today's presentation, there is a chance we can identify new or novel protective antigens. The other thing is how can we deliver antigen. We know the live attenuated approach probably is not viable nowadays, so what are the other choices for us, especially in light of induction of several immune response, like presented by Steve right before my talk, and also, of course, today we talk about what is an acceptable animal model because many

modalities relate to what model you are looking at.

The safety part I thought is also very important, because that relates to how do you protect, produce? Produce vaccine, it is not what type, live attenuated or not, it is really standard what you can really have a well-defined product to go into human trials rather than you know the antigen.

Also, how do we select additional antigen? And then finally, how do we administer into potential human population.

So, our strategy at U. Mass was based on the following premises. One is built on our previous experience on design of novel vaccines. Our focus was not on the modality of what type of vaccine, rather identify immunogenic antigens, because a vaccine is the business of antigen. We need to pay more attention on that.

The second part, is how do we deliver, what is the technical approach. We focus on the subunit based approach, but subunits in my laboratory expanded to include both the recombinant

protein, as well as DNA as the subunit.

Actually, DNA, to me, actually is the best approach to develop subunit vaccine. You can bypass many technical difficulty when you deal with a protein antigen.

Then, finally, we further tried to use efficient system to screen for new protective antigens.

So, let's show you the first, just an example, this study. Actually, most of that already been published in recent issue of Vaccine, but I just want to give you more detail here.

So, in this first study, we included three potential antigens: V and F1, that is well known as a potential protective antigen; we included Pla, Jon Goguen has been one of the pioneers showing the pathogenesis of Pla, so we also want to see whether this can function as a new protective antigen.

As you may read in literature, our colleagues at UK have done work in vaccine, especially use the B antigen. They have used a gene gun, which is my favorite approach. They also

optimize, they also use the DNA protein. Those are all the approaches I like, however, they still did not see -- they reached some level close to recombinant protein.

So, one trick they have not done, which I do a lot in my lab, is look at the leader sequence here showing as a black box. The difference between bacteria antigen and the DNA vaccines is the DNA vaccine has to be expressed in the main system, and for the V antigen, they don't have a putative leader sequence.

But we know that, when you have an antigen, a special post-secreter, antibody antigen, you need a secretion. So, that is why I am making some kind of F1 antigen, is very immunogenic because it secretes.

Also, Pla has a hydrophobia reason. We don't know whether that is a leader or not, but still it is a popular strategy or at a leader sequence. Actually, we find a very different type of a response.

So, here is the immunization schedule,

very simple. Every 4 weeks or monthly, we give a DNA immunization. We use a gene gun. Each animal, we give 6 micrograms of DNA. I just want to emphasize this is not an optimizing. You can see the protective efficacy of our approach.

Then, we wait a long time. That is not because we designed it that way, because our 303 lab was not available, we have [inaudible], and so on and so forth. Finally, we have a boost and challenge, and later I can show you, this actually not relevant, we can shrink that, earlier challenge.

Here is the RB antibodies. I get a response by ELISA. You can see the V works very well. F also works. The tPA maybe improve a little, but not too much from the binding antibody, but the Pla was completely negative. That shows you Pla is not immunogenic by this design.

Then, I will just go quickly to the key part, so you can see that for the tPA of wild type, the tPA actually have more secrete--they have early rise. After one immunization, you see very tight

antibody response, but after immunization 2 actually, they reach very similar level of a response, so the binding antibody, tPA-V or wild type of V, they are very similar.

However, the interesting thing is here, better protection. This is the first time you study, we use a 5,000 CFU, which is equivalent, about 100 LD50. You can see that we see three patterns, three antigens. This is the V group here, this is the F1 group, this is the Pla.

Pla has no protection at all, the same as the control. We see in 3 to 5 days all animals without exception all die using the strain 100. That is the one Jon Goguen used for many years.

You can see the F1. Whether you use the tPA or not, they are in similar range of partial protection, and then the real interesting part is the V antigen, very clear cut. With the tPA, no exception, all protected. You can follow that with 2 weeks or even longer. The wild type, as we expect from V antigen, there was protection, but a partial, the same as the F level.

So, this confirmed previous work that V can protect. So, the next question we want to ask, can we give a higher challenge dose. You can see here, this is the same 5,000 CFU, this is the 20,000, and this is 80,000, which is about 300 LD50. This is all intranasal challenge, by the way, and under 50 microliter, I agree with a comment earlier, what we do intranasally is we anesthetize the animals, you draw up the Y. Initially, Jon was a little bit suspecting whether that would work, but if you see it once, you know that they will draw up everything, very reliable technique.

So, here you can see that after the challenge dose, 90 percent protected with tPA. The wild type at the baseline is about 20, and then when we go higher dose, 20,000 CFU, you can see about a 80 percent, then, 70 percent.

If we put all the data together, just compare here the control group, all the animals now survive, and also I want to comment here, the size of animal group were increased to 10 animals, so 90

percent for--for the tPA-V at 500,000, and 80 percent, that's 20, and 70 percent or 80 percent.

Unfortunately when we transfer the 5, this number lost. Here, at the two dose, all significant. If we combine all 3 together, you can see the p-value is very, very significant, so clearly the wild type and tPA-V are very different qualitatively.

So, the question is what happened here, they are all V antigen. They should be the same V antigen. So, it is very interesting. We want to prove our antibody, then, we find something very interesting, that is, here, with the tPA, they form the dimer or tetramer. For the wild type, you don't see that, and they have less secreted. Because of time, the reason I am not showing the other--if you look at our paper, you will find if we over-express or produce here, this is wild type, you can still see just a band, don't form oligomers.

The binding is very strong for V antigen, so that is why when you make a fusion protein,

sometimes [inaudible], you will see that, you will reduce it back to single band. Prove that.

The next question is interesting. So, we look at what are the subtypes as a simple way to measure Th1, Th2, as we discussed earlier this morning. You can see that because we use gene gun, so we can see a predominant IgG1, a pH of 2, as we expected from a protected antibody.

However, the simple fraction of the IgG2A, which is representing Th1, actually was increased with tPA type rather than the wild type, so suggesting somehow when we change the leader sequence, the 40 of the protein is different, as we see from the previous western gel. So, there is some confirmation difference.

We know that the leader sequence is actually very critical after translocation, protein differences. We know the so-called immune suppressed function, probably is the N-terminus. So, whether that 40 actually affect that, we don't know. So, that is a very interesting question.

Given the time, I will just quickly show

you some other data very quickly, just one minute.

So, we also look and use the same technology to quickly screen like we are doing here, we look at YopD, YopB, and YopO, because you can see, sometimes a bacteria antigen has two hydrophobic domains or just has one, so we made all kind of antigen engineer, remove them or not removing, add a leader or not, then, you can see the result here.

So, with YopO, the wild type, they were mainly intracellular, but we make a tPA and allow to secrete. With YopB, unfortunately, again, Microsoft shifted here, so you can see here, we have N-terminal here, very well expressed, we can selectively express certain domain, [inaudible] or you have the tPA with no change at all. You can see if you remove the hydrophobia region, you will actually induce a secretion, improve the immunuogenicity, and the same thing here with YopD, so we are looking at whether this antigen can provide any protection. I can tell you briefly, basically, we did not see a major protection.

However, what I want to show here with this technology, you can quickly screen many antigens in a reliable, protecting model without going through very complicated protein production process.

So, this is our strategy for the future study. We believe the DNA or DNA-plus protein is a very viable approach to generate emerging vaccines as most people agree in this audience, and we want to have proof of efficacy, protective antibodies, plus proof of cell-mediated immunity. Whether that is CD-4 or even CD-8, we don't know yet, and also we use DNA as a protein, as a technical protein.

We believe that this is a safe and very easy to administer, and the most important thing, if we use subunit and DNA in the future, the plague vaccine can be mixed with other biodefense--so the soldiers, when the go to field, they don't have to receive 20 needle sticks. They can use probably one or two.

So, I will stop here. Finally, I want to thank my collaborators. At the top are the people

from my laboratory. I want to thank my colleague, Jon Goguen, who gave all the guidance and without him I don't think we would make such a program.

Thank you. I will stop here. [Applause.]

DR. LINDLER: Any questions?

Thank you.

The next speaker is Kathleen McDonough from the Wadsworth Center speaking about profiling differential gene expression in Yersinia pestis as a tool for vaccine target identification.

Profiling Differential Gene Expression in Yersinia Pestis as a Tool for Vaccine Target Identification

Dr. Kathleen McDonough

DR. McDONOUGH: I want to start by thanking the organizers for the invitation to speak today about a project that is very new in the lab. Unlike the plague doctors of the Middle Ages, we certainly have a tremendous number of tools, particularly most recently the availability of complete genome sequences, but I think although we are rich in these technologies, we are also, as we

have seen today, still fairly poor in answers about what makes plague bacilli really tick.

So, what we are interested in is identifying some environmentally regulated plague-specific gene products that may also be useful for pathogenesis for the organism and, from our perspective, for diagnostics in vaccine design.

Classically, in terms of the Yersinia, DNA relatedness has been not a good indicator of biological similarity or at least in terms of pathogenesis, and so our approach has been to think about looking instead at expression profiling to get at some of the more unique pestis attributes, and, in particular, as we have been hearing all today, the disease that pestis causes is certainly very different than the disease of either the other enteric pathogens, the enterocolitica or pseudotuberculosis, and, of course, only Yersinia pestis is transmitted by fleas.

So, in terms of thinking about expression profiling, the most immediate choice we had to make was protein versus RNA, and we have chosen the

proteome approach for a couple of reasons listed here.

In particular, protein is a more final product than RNA, and very importantly, we think that this then allows us to get to posttranscriptional regulatory products that we think may be important particularly for plague.

Protein also has more direct potential than RNA as a direct vaccine target, and, of course, we have some additional advantages to doing proteins over RNA in that we can fractionate our samples, and so on, before we look at them if we want to get, in particular, for vaccine type of development, secreted or membrane-bound antigens.

So, back onto the idea of posttranscriptional regulation and thinking that it may be something of particular importance for Yersinia pestis. A recent paper out of Bob Perry/Jackie Featherston's lab, the HMS phenotype that is critical for blockage of fleas and therefore transmission by the natural plague rodent/flea route is posttranscriptionly regulated,

and an older paper out of Brubaker's lab, it is shown that some of the Yop routines are degraded by the plasminogen activator on the pestis and plasmid at least in vitro, and whether this happens or is important in vivo or not has not been followed up.

I am going to skip the sort of technique slide there. I think most folks are pretty familiar with 2D GEMS or 2D GEL electrophoresis in mass spectrometry, and just move on to some of the applications.

This is actually a study from a different project in the lab on TB that illustrates the point of how 2D GEMS can be particularly useful for identifying posttranscriptionally modified bacteria, and this is just looking at differential protein expression in a vaccine strain of TB, and the only important things to get here are that with some of these identifications that are shown here, the two most prominent differentially expressed one, this Number 7, the PE PGRS6, and the GRO-EL2 are both actually posttranscriptionally modified, as well as transcriptionally differentially

expressed.

Those are the two that are shown here in blue, and you will notice that at the protein level, GRO-EL2 is differentially expressed or induced 10-fold in the one condition over the others, but only 2.3-fold at the RNA level. Likewise, 27-fold deduction at the protein level for this PE PGRS protein, and only a very minor or relatively minor increase at the RNA level.

So, the 2D-GEMS is particularly useful for looking at the total protein effect. I should say that of those two prior proteins, one of them is a protease cleavage event, and the other is a lot more likely a translational regulation.

So, the other thing that we would also get that wasn't done, if there is any kind of other protein modifications, methylations or phosphorylations, et cetera, they will ship them in gel, and they will come up in this kind of analysis.

So, in thinking about what kind of regulatory conditions would be interesting to look

at, a first temperature in calcium or the other really classical regulators for plague, as well as the other Yersinia.

Iron, as well, a tremendous amount of good work has been done on this, particularly out of Bob Perry's lab, but the rest of environmental conditions have not been well addressed in

rsinia, and in all of them, or each of them may also have a role mammalian host particularly with respect to the time they may end intracellularly within macrophages as has been alluded to veral times today.

So, the data that I wanted to just show you today has to with looking at hypoxia, and one of the reasons that we chose poxia is because in other organisms, hypoxia has been a very good gnal to look at to identify genes that may be induced or gulated within the macrophage.

So, in particular, the thing to key in on here is that in rms of oxygen in atmospheric air, it is very high levels. Also, the lumen of the lungs, the oxygen is also going to be very gh,

but then once you are inside a cell, the oxygen will drop quite a bit, down to about 2.6 percent. Another thing that happens in the mammalian host that is different from what is in e environment is that the CO 2 levels in the environment are very low, but almost anywhere you go, in a cell or out of a cell, within a host, is also CO 2, so in our hypoxia nditions, when we modulate the oxygen, we also include CO 2 when we are thinking about mammalian conditions.

So, what this has shown here is just a little profile of some proteomes. On top is Yersinia pestis, and on the bottom is pseudotuberculosis, and either on the left, ambient air conditions or hypoxic conditions, which were 3 percent oxygen and 5 percent CO 2, and the things to clue in on here, the real question we were asking, are there differences between pestis and pseudoTB in this condition.

So, the boxes are showing proteins that differ between pestis and pseudotuberculosis. So, for example, here you have got that little doublet.

You have got the top guy here. He is missing here, he is missing here, and so on.

Also, the circles are going back and forth and showing intraspecies differences in pestis or in pseudoTB, and there is others that are not marked here that are lost in the translation.

But the other things to keep in mind is that we use strains to try to match, mostly for the chromosome, and so that we wouldn't have as much interference in terms of differences from some of the extra plasmids, and so both pestis and pseudoTB were both LCR-minus.

The pseudoTB isolate, we used is a serotype I that has the high pathogenicity, and also the pestis that we use is 10-plus, so it also is missing the pestis in plasmid.

In terms of extra DNA that we know is there, the PMT1 plasmid is present in pestis, and not pseudoTB. So, what we will move on with is also looking, as well, at the different contributions of each of the different plasmids in terms of regulating chromosomal genes.

This just shows a later time point. The time point I just showed you was one hour in hypoxia. This is now 24 hours in hypoxic conditions, and this is just pestis, and this is showing a number of differences between the ambient protein expression versus the hypoxic protein expression.

So, I have shown you so far, or what I have shown you, all I am going to show you today, is essentially the 2D-gel electrophoresis. We think it is a useful approach for identifying pestis-specific responses to the environment.

The pestis proteome does change in response to the hypoxia, and pestis and pseudoTB also respond differently to these conditions. Where we are going in the future and currently is that we will analyze the response of pestis to see the additional environmental conditions that I mentioned. People have ideas about other things or in terms of prioritizing things.

We are certainly interested in hearing them. In addition, what I didn't show you is any

metabolic labeling differences, it was all just Steady State approaching comparisons. With metabolic labeling, of course, we see lots more changes although they are harder to follow up in terms of the mass spec. protein IDs.

In addition to doing the 2D-GEMS, another mass spec. approach is ICAT technology or the isotopically coated affinity tags. We are doing some of that, as well. It essentially bypasses the 2D-GELS, and it is a very complementary approach to the 2D-GELS because you are able to analyze kind of different sets of proteins, as well as some overlap.

Then, of course, for the future, we will move on with the proteins that are identified and characterize them with respect to regulation and function, and their potential as vaccine or therapeutic targets.

The people simply I mention here would be Michael Gazdik had done the TB-related gels that I showed you, and David Schaak did the plated gels.

Thank you for your attention.

[Applause.]

DR. PERRY: Bob Perry, University of Kentucky.

Kathleen, is there a difference in the growth rate between your atmospheric and your hypoxic strains? Does it really drastically change the generation times or are they growing about the same rate?

DR. McDONOUGH: We haven't analyzed that really carefully yet, so I can't say total, but there was nothing really dramatically obvious in terms of culture densities, but this is the kind of thing we used to go do.

DR. PERRY: I just sometimes think we need to sort of monitor that and then see if some of the changes are not due to oxygen, temperature differences, but growth rate differences, and do that by adjusting your growth rate with other deficiencies.

I think there have been some microarray studies where they haven't taken that into account, and you see a whole bunch of weird genes that are

iron regulated, but I am wondering if they are growth rate regulated instead.

DR. McDONOUGH: Nothing looked obviously different. They didn't seem really challenged. The other thing is they were only in for an hour.

DR. LINDLER: Have you been able to map those to specific regions in plague or pseudoTB, where those gene products are coming from?

DR. McDONOUGH: Not yet. Next on the list. One of the things we typically do. You get a lot of variability in terms of gel-to-gel is typically very consistent, but in terms of biological repeats, and we have learned from experience that before we go on and identify things by mass spec., we end up setting up really rigid criteria, so that we like to have at least three biological repeats of proteins that are reproducibly changed before we move on and do the I.V.'s. So, that is still in progress.

DR. LINDLER: Thank you.

The last speaker is Leah Scott from Defence Science and Technology Laboratory. She is

going to speak about the marmoset as an immunological model for plague.

The Marmoset as an Immunological Model for Plague

Ms. Leah Scott

MS. SCOTT: Good afternoon, everybody. Thank you very much for your forbearance at the end of a long but productive day.

I would just like to spend a few minutes highlighting some issues that I think are terribly important to us all. Particularly, we have heard from Louise about the importance of nonhuman primate models in this area, and this is an option that I just want to raise with you. We will be around for the rest of this evening and tomorrow if you want to discuss things in greater detail.

For those of you who may not be familiar, familiar with the marmoset, here they are - small, new primates 350 to 450 grams. I will just say very quickly, this is background, what we know about immunologically, I will allude to work in progress, and finish off with some remarks about other sources of information.

We know, worldwide, the common marmoset is becoming much more popular and has been widely used in many areas of research including, as it says there, including a number of fundamental applied research areas in regulatory studies in diverse areas, particularly in neuropharmacology, behavior and toxicology. We know about those issues. They have been around for a long time.

But specifically in the context of the world which many of us in this room live, in the UK, the marmoset has been extensively used to elicit the effect of nerve agent poisoning, and it continues to be absolutely pivotal to us in bridging guinea pig studies to human studies when we are talking about the development of nerve agent pretreatment and therapy.

What our plans are for the future, we are looking at marmosets. We haven't done plague in these animals yet, but we have plans to do so in the not too distant future. We aim to characterize the model, understand its relative strengths and weaknesses, which is a fundamental approach that we

have right across our work here, and we aim to do that, as I say, in the next six months onwards.

We have been involved in characterizing the marmoset as a model in immunological studies because of the middle bullet there. The marmoset has been used in our laboratory in a very high profile study over the last four years to look at the effects of multiple vaccinations in the context of Gulf health.

Previously, it had not been particularly well characterized as a model in such studies, and we had to build upon one or two case studies and build up the toolset, so that we can understand the impact of vaccination in this model. We are now in a position to do that.

The big issue, of course, with the marmoset is because of its small size, and its incredible productivity in terms of laboratory management and captive breeding, marmosets tend to have twins or triplets twice a year, and it can be used from age 11 months to 12 months onwards.

So, those of you familiar with large

primate studies will see that there are enormous benefits to be gained from that. Moreover, their captive management is relatively easy, and that includes in high levels of biocontainment. So, certainly worth considering in this context.

This is just a summary of what we can do at the moment. In view of the lateness of the hour and the short time that I promised to talk for, I shan't go through it all.

Suffice as to say we have the toolset. I have some exemplar data. Come and see me afterwards or tomorrow, and we can discuss those issues. Just wanted to flag up the big issue.

Many of you in this room, all of you in this room will know our existing plague team, but these are a few other folks, some of my other colleagues at DSTL, what I would call the Parent Marmoset Immunology Team, who have been looking at marmoset vaccine studies in the context of Gulf health.

Gareth and his team would be very pleased to help. I would also like to draw your attention

to the European Marmoset Research Group, which was founded more than 10 years ago now, and is developing as a very strong information base, the discussion of such issues, and more recently, the development of the Marmoset Research Group of the Americas. The web site address is there.

Thank you very much for your forbearance and will look forward to talking to you.

[Applause.]

DR. LINDLER: Any questions?

I would like to thank the organizers and I will turn it back over to them. Thank you.

[Whereupon, at 4:48 p.m., the proceedings were recessed, to reconvene on Thursday, October 14, 2004.]