SOCIETY OF TOXICOLOGY

CONTEMPORARY CONCEPTS IN TOXICOLOGY SECTION

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C O N T E N T S

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

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

1A MS. MILLER: I'm your lead-off speaker this morning. I am Margaret Miller. I am with FDA's Office of Women's Health, and our office is located in the Office of the Commissioner. And our mission is to serve as a champion for women's health, both inside and outside the agency.

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

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

[Laughter.]

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

Now, while we recommend the participation of women in clinical trials, we do still have concerns about fetal safety. And so the recommendation does not extend to pregnant women. So regarding the participation of pregnant women in clinical trials, this is really limited to those products which are intended to treat a condition that occurs only in pregnancy.

Well, this leaves us with a problem. Because while we are not including women in clinical trials except for those cases where it's used to treat pregnancy, we know that women get sick. Women get influenza while they're pregnant. And treating a pregnant women often confers benefit not only to her, which is our office's concern, but to the developing fetus as well. But yet, at the time of an approval we don't have information on fetal safety, or even on what health benefits or differences that product might have in a pregnant woman versus a non-pregnant woman.

To add to the problem, we have the fact in this country that about half of all pregnancies are unintended; which means many women are having therapies and different treatments without knowing they're pregnant. And at the time that they realize they're pregnant, they go back through their mind and go through all those things they've done for the past month or so. And they come in and they say, "How will these activities affect my baby?" And that's a big question for them.

So when a clinician is trying to treat a pregnant woman or a woman of childbearing potential, they really want to balance the health benefits of a product versus the safety concerns for both the fetus and the mother. And in order to do this, the agency has recognized that this is an area where we really need to do a better job in providing clinicians and women with this type of information.

One of the activities that the agency is undergoing is an effort to revise the labeling section of our products. And this has been an ongoing concern that the health care community has brought to the agency: that the label--the way it's formatted and the information that is imparted--

does not provide them with the type of information they need to make clinical decisions. So that is an ongoing effort.

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

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

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

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

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

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

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

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

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

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

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

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

Finally, or next, if the research is designed to meet the health needs of the mother, and the risk to the fetus is minimal or the minimum that we can obtain with the study, then the maternal consent alone is necessary.

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

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

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

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

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

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

And so we have a list of all ongoing registries that are for medicines that women need to maintain their health. And it is to encourage their participation. And we also have a "Contact Us." So if you have a registry that you would like to have included on our list, you can send us an e-mail and we'll incorporate that into our registry list.

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

And I think--and we heard some of this yesterday--that even if we had registries for everybody, we're limited as to the type of information that you can get from a registry. You're not going to necropsy those babies and do lymph nodes. You know, it's just not going to happen.

So if you don't know what you're looking for in a registry, you're going to maybe look for major malformations. There's problems with long-term follow-up. Really, you need the animal models to give you the signals, even to design a good registry: What should we be looking for?

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

And certainly that is the challenge for this group. And after the discussion yesterday, I'm sure you're up for it.

So I'll turn it over to Marion.

[Applause.]

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

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

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

We will make available the presentations, the slide presentations of the speakers, following this meeting of the SOT, once they have received all the slides from the speakers. We'll make them available about two weeks after this meeting. And I think we're also thinking about having an evaluation form that you can then fill out by e-mail.

So having said that, and I hope I didn't--No, I forgot a lot of things. I was told to thank all the speakers and panel members yesterday for a very fruitful and helpful discussion. That has been tremendously helpful for FDA, and we think we have an idea really how to actually begin to think about guidance. Let's be careful.

And I also wanted to thank again, as Karen Midthun did yesterday, the SOT, and especially Shawn Lamb and her staff, for making this a very smooth, easy-going workshop. So thank you very much, to Shawn and her staff, for helping us with this.

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

REPRODUCTIVE TOXICITY ASSESSMENT OF PREVENTIVE VACCINES

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

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

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

The guidance was published with the intent to provide sponsors with information regarding assessments of the reproductive toxicity potential of preventive vaccines that are indicated for maternal immunization, and to target populations that would include females of reproductive age. This document was generated and written when there was relatively little experience with performing reproductive toxicity assessments for these types of products. And there was virtually no scientific literature to really assess and address this issue.

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

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

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

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

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

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

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

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

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

Now, most vaccines that are currently licensed in the United States are not indicated for use during pregnancy. But depending on the vaccine, vaccination programs do frequently include pregnant women. For example, as Peggy addressed, the inactivated flu vaccines are often recommended for use in pregnant women in their second and third trimester of pregnancy. Those women were at special risk for serious consequences from the flu.

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

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

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

And there are a number of controlled clinical trials that have been conducted. And they provide evidence that maternal immunization with at least inactivated vaccine antigens, including haemophilus influenza type B and pneumococcal polysaccharide vaccines, appear to be well tolerated in the pregnant women and in their offspring.

But I think what needs to be stressed is that these studies were usually not powered or designed to detect rare adverse events, or to assess long-term follow-up of the offspring.

Even though there may not be hard evidence of reproductive toxic effects in humans caused by the use of currently approved vaccines, when assessing the preclinical and clinical safety of a candidate vaccine regulatory considerations take into account not only past experience, but also theoretical concerns.

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

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

The current situation is, as Peggy pointed out, unless the vaccine is specifically indicated for maternal immunization--that is, indicated for immunization of pregnant women--no data are collected regarding the vaccine's safety in pregnant women during the pre-licensure phase of a vaccine.

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

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

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

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

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

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

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

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

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

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

And this rule would not only eliminate the current pregnancy categories, but the rule would require labeling to include a summary assessment of the risk of using a product during pregnancy and lactation. And it would require a broader discussion of the data--and that is animal and human--that would underlie the evaluation of risks associated with a product.

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

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

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

You should note that industry comments represent several different points of view. And there are going to be apparent contradictions. But I decided to present those to you, to give you a true representation of the various issues and concerns and questions that have been raised. And I think this will certainly spark a lot of discussion, but I would like actually for you to hold your comments and questions until this afternoon, because this is when we are looking at the different issues.

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

If you have clinical experience that is derived from immunization of pregnant women, then this experience or the data and the outcome may be considered for any potential application in the design of the reproductive tox study. All data that you may have from acute or repeat-dose preclinical tox studies should be reviewed for their possible contribution to the interpretation of any adverse developmental effect that you may observe in the developmental tox study. An example provided was fetal toxicity secondary to maternal toxicity.

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

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

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

And we had also recommended to extend the stages "C" and "D" evaluations to also look at the period between birth and weaning, defined as stage "E" in the ICHS5A document, so that mother and offspring can be followed postnatally.

So what did industry have to say? Actually, the majority of comments supported that, in principle, developmental tox studies for preventive vaccines are needed for vaccines that are indicated for maternal immunization and females of reproductive age; and that thus, efforts should be made to assess the risks of vaccination during pregnancy in animal models.

At the same time, however, we did receive comments questioning the relevance of developmental and reproductive tox assessments in animal models for preventive vaccines.

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

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

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

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

Turning back to the guidance document, the section on immunological parameters and follow-up, the guidance states that the reproductive tox studies should be designed to include the detection of antibody production in the pregnant animal, and to also look at the feasibility of antibody transfer from the pregnant female to the fetus through antibody measurements in the fetus and newborn.

We also thought that the antibody response in the fetus should be studied, looking at presence, persistence, and effects; including potential cross-reactivities with the antibodies induced in the pregnant mothers with fetal tissues.

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

Industry says that in general there is agreement that it is important to demonstrate an immune response to the vaccine in the dam, to demonstrate exposure. And the ability to detect an antibody transfer from the dam to the newborn was viewed as a key issue by some. And the suggestion was made that the proper species for a developmental tox study be validated in a preliminary study with only immunological endpoints.

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

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

One comment questioned the "appropriate immune response" in an animal model, as antibody generation would be only one factor. Cytokines, cell-mediated immune responses could also result potentially in toxic effects; each with their own specific time lines.

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

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

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

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

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

Furthermore, a comment was made that body weight is the best indicator for pre-weaning developments, and functional studies are not commonly conducted in pre-weaning pups, due to the limited repertoire of responses and difficulty in the quantitation of those responses.

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

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

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

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

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

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

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

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

We had only one comment from industry regarding immunization schedule and exposure, and that was loaded. The relationship of dosing to developmental timing will be one of the most difficult aspects in designing developmental tox studies.

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

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

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

The guidance document further states that if there is no relevant animal model, then reproductive studies should be done regardless, to assess the intrinsic potential of vaccine antigen. And I think we need to really discuss this this afternoon: what to do if we don't have a relevant animal model available to us.

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

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

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

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

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

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

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

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

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

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

[Tape Change.]

1B DR. GRUBER: --that is specifically indicated for immunization of pregnant women would need to be available prior to the initiation of any clinical trial that would enroll pregnant women. But if you have a vaccine that is indicated for adolescents and adults, it may be okay to include women of childbearing potential into clinical trials without reproductive tox studies, provided that appropriate precautions are taken, such as pregnancy testing and use of birth control.

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

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

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

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

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

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

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

And in random order, the questions are:

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

If you focus on immunological parameters, what should be focused on? What should be assessed? Are antibodies enough? Do we need to look at cell-mediated immune responses, cytokines? And how far should we even assess potential interaction with fetal tissues? Should there be kinetic assessments?

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

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

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

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

How do we deal with the dosing?

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

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

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

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

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

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

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

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

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

[No Response.]

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

[Applause.]

DR. GRUBER: Well, it is a great honor to introduce to you the next speaker. It's Dr. Richard Insel, who is the Director of the Center for Human Genetics and Molecular Pediatric Diseases in the AAB Institute of Biomedical

Sciences. And Dr. Insel is the professor of pediatrics and microbiology and immunology at the University of Rochester School of Medicine.

His early research focused on the development and immunogenicity of haemophilus influenza B conjugate vaccines. And he was part of the research team that developed conjugate vaccines for infants, which of course, as you know, have eradicated infant bacterial disease from invasive haemophilus influenza and eliminated the most common cause of meningitis in children in the United States.

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

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

Ladies and gentlemen, Dr. Insel.

[Applause.]

HUMAN T AND B CELL DEVELOPMENT

PRESENTER: RICHARD INSEL, PH.D.

UNIV. OF ROCHESTER, SCHOOL OF MEDICINE

DR. INSEL: Marion, thank you.

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

What the first slide shows is, I like to think of the immune system as composed of two really major components: what we call "innate immunity," and adaptive immunity. And innate immunity exists to immediately and quickly recognize that the host has been invaded, that there is a danger on board, there's something foreign on board; and responds quickly to that response with either a cellular response, as shown here with, in this case, antigen presenting cells, APC's--one example of which would be professional dendritic cells. And they respond to contain that insult, and will invoke an inflammatory response to contain and destroy that insult.

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

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

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

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

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

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

So on the left, if we look at B cell development, which in the adult is going on in the bone marrow, we have initially a progenitor B cell that gives rise to a pre or precursor B cell that has cytoplasmic "U" [ph], that then gives rise to an immature B cell which has on its surface IgM, and then giving rise to the mature B cell which has IgM and IgD. That cell then leaves the bone marrow to move to the periphery.

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

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

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

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

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

At the next stage when it moves into the B cell stage of things, extinguishes T cell development, one has what we call B cell lineage specification associated with onset of expression of new transcription factors.

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

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

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

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

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

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

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

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

Mouse development occurs much later in comparison to human development, in comparison to the total length of gestation of approximately 20 days. One doesn't see fetal liver hematopoiesis or lymphoid development until about halfway through the gestation period, and one doesn't see bone marrow development until three-quarters of the way through gestation; in contrast to the earlier development in man.

Now, this transition from fetal liver to bone marrow for definitive hematopoiesis as well as lymphoid development is not as simple as this. But as shown on the slide, it's really a continuum. What one has is, approximately at six weeks of age, the onset of fetal liver development, of lymphocytes and hematopoiesis, which gradually peaks at about three months of age, and then tails off and is extinguished by approximately 30 to 35 weeks of age. It is gone by the time of birth.

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

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

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

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

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

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

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

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

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

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

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

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

In the second trimester, by 15 weeks, the percentage of B cells in the spleen, lymph nodes, and blood is equal to what we see in postnatal life. And so you can see how this is very early in the development we've acquired now numbers very similar to what is happening in postnatal development. At 18 to 20 weeks, we see primary follicles in secondary lymphoid organs, such as lymph nodes in the intestine. A few weeks later, we see primary follicles in the spleen. And then what we see in the third trimester is loss of lymphopoiesis in the fetal liver, and the bone marrow becomes the primary site.

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

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

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

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

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

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

And the third trimester is associated with increased cellularity in the thymus. We see some increased diversification, with increased CBR3 size. And we see increasing cells in the periphery. So the third trimester is primarily associated with expanding those cells that are there at that second trimester.

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

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

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

So we've just looked at cellular contribution in the fetus to immune development. What I want to turn to now is, as all of you appreciate, the fetus also is bestowed with maternal immunoglobulin. Of the isotypes, IgG is the only isotype that crosses the placenta. Passive transport begins in the first trimester, quite early. Active transport begins in the second trimester, and it picks up in activity near the end of that second trimester.

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

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

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

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

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

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

Obviously, Rh incompatibility, ABO incompatibility, antibodies to platelets, can give rise to thrombocytopenia, and antibodies to white cells can give rise to leukopenia-- very well known reactions. And newborns born to mothers with myasthenia gravis or thyroid disease may also develop those diseases, such as myasthenia or thyroid toxicosis. And maternal antibodies can also cause membranous glomerulonephritis in the offspring. So it is something that we must also keep in mind.

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

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

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

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

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

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

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

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

And if one looks at cord blood, one can culture cord blood lymphocytes--and specifically cord blood T-lymphocytes--with antigens from these parasites, and show specific T cell proliferation. One can show that those T cells not only proliferate, but will produce cytokines. And they will produce cytokines, not just TH1 cytokines; but will produce both TH1 as well as TH2 cytokines.

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

Last, with allergens, both with indoor as well as outdoor allergens one can demonstrate, using cord blood lymphocytes, T cell proliferation. One can demonstrate proliferation of not just naive, but memory T cells. And one can demonstrate that those T cells can make multiple cytokines, often of the TH2 variety, IL4, IL5, IL10, and

IL13. And one can generate even allergen-specific T cell clones, and show that they have the ability to generate these cytokines.

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

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

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

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

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

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

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

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

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

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

Well, the bottom line is that on the human side there's not a lot of data to suggest that this is occurring. When looked at for haemophilus influenza B, influenza virus, at

Group B streptococcus, at pneumococcus, there is no good evidence that it occurs.

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

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

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

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

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

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

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

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

There may be restricted repertoires early on in life. And we do have this age-related hierarchy of responses. As we know, responses to polysaccharides don't occur until usually two years of age or later.

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

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

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

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

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

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

As I have noted, the fetus can generate immune responses to congenital infections, to allergens, as well as to protozoan antigens. The fetus can acquire maternal IgG, and it's something that one will have to keep in mind. And last, the human is not equivalent to the mouse.

I thank you for your attention.

[Applause.]

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

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

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

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

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

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

DR. INSEL: Yes. I can't. You know, there are different populations of NK cells. I don't really have the data. But if you come up to me later, I'll be glad to look it up and send you that. But I can't give you good data.

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

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

Since I have left the organization, I have been a consultant. And just so people know where I stand, I have consulted not only with the government, but also with manufacturers, including Aventis Pasteur and Merck.

Nobody has told me what I should say, I would hasten to add. So these are only my own thoughts. And I kind of worry about--

[Tape Change.]

2A MR. PARKMAN [In Audience]: --one product that has had the potential for bad reproductive toxicity, and that was German measles vaccine. And I'm surprised there wasn't more mention of that. That was a long time ago, of course. It was in 1969.

The studies that were done that suggested that the vaccine was not reproductively toxic were done almost entirely by the Division of Biologic Standards. And the results in animal models, the monkey was selected. I mean, we kind of looked back to epidemiology, and saw what the disease did. We used monkeys as a model, because they are kind of close to man. We developed a model for the disease.

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

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

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

I would only counsel that the FDA be careful. It takes a lot of time and effort to do these studies under GMP, that can take away from what people can do on other things.

I think it would be worthwhile some time to review the reproductive toxicology and the toxicology of products that have been approved before, not only rubella but perhaps other topics. If thimerosal is a big issue, maybe it would be worthwhile to look and see what is being done now to look at that issue.

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

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

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

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

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

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

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

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

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

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

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

[Recess.]

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

MATERNAL IMMUNE SYSTEM STIMULATION

AND EFFECTS ON FETAL TERATOGENESIS

PRESENTER: STEPHEN HOLLADAY, PH.D.,

COLLEGE OF VETERINARY MEDICINE,

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

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

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

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

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

The beginning of this concept was in 1990. Note the journal, the "Journal of Experimental Medicine." The group that published in this journal was a Japanese group, primarily involved in cancer research. The head investigator of that lab's name was Nomura. Why they shifted into teratogenesis for a brief time, I'm not sure. It wasn't talked about in the paper. But their data were very interesting, and this caught our attention, actually about 1991, when we first saw these data.

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

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

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

And you can see in this case that these are not all the data from their paper. The paper was quite rigorous. Many replicates were indicated. Again, the journal is a good journal, with a powerful peer review process. We had to assume that this was a well done report. And indeed, when we validated in our own lab, we found the same results.

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

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

And the birth defects were reduced by the Pyran copolymer immunization from 35 to 20 percent, approaching a two-fold reduction. A physical agent was also investigated, X-rays. Tail defects were the predominant defect. And we see here a two-fold reduction in that defect.

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

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

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

In this experiment, the first experiment, we had enough animals that we could use a vehicle exposed control. In subsequent experiments, we've used immune stimulated controls. The immune stimulation has not produced undesired effects on the pregnancy. In fact, it appears to have some desired effects--decreased resorptions, and so forth--in addition to the reduced teratogenesis.

This particular experiment was the only one that I've conducted where a vehicle exposed control had a spontaneous defect. That's why there's a little bit of height on that column there. We had one exencephaly in that experiment.

These inbred mice are a bit harder to breed and a bit more expensive to breed than outbred mice. So our next question was: What happens if we do this in an outbred animal? And these are ICR--Again, a CD1 mouse, an outbred mouse. And we repeated the same experiment: methyl nitrous urea, Pyran copolymers, the immune stimulation given, IP. And you see a similar profile here, in terms of reduction of the birth defect, the digit defects.

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

And the outbred animal also has responded better to the immune stimulation, in terms of reducing the birth defects. Here we have about a 30-percent decrease; and here we've got more than a two-fold decrease in birth defects, digit defects caused by this immune stimulation. So we have moved to outbred animals, and now that's primarily what we use.

We also--Well, that's a hair trigger there. Let's see. Okay. Evaluated the same defect caused by another chemical. This is urethane. Again, we've done this with different immune stimulants, and under different conditions.

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

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

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

We evaluated cleft palate also by urethane, and probably have spent most of our time there, in terms of trying to understand mechanisms by which this immune protection might work.

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

We found early on that when we dose with urethane--this was at a relatively high level of about 1,000 milligrams-per-kilogram--on the morning of day ten of gestation, we could create cleft palate in about two-thirds of the fetal mice. Also, we noted that the cleft palate we produced was of two phenotypes, without much of an integrate in between. We have what we called a "wide cleft." I hope you can see that from back in the seating. And we have what we called a "narrow cleft," a more slit-like cleft. And it is probably something we can explain fairly readily by precise timing of closure of the palate with the chemical exposure, but we did have these two very different phenotypes. And we characterized them with the immune stimulation, as well.

Different stimulants were being used in the lab. I tried to get away from BCG because, while it was very effective for us and worked well, contamination of laboratory personnel might result in a positive TB test, and we don't need that. Actually, I switched to BCG because I ran out of Pyran copolymer; contacted the Hercules Corporation that produces that, and they indicated, "You know, we stopped over ten years ago. And what we've been supplying has been on our shelf, and that's gone now." So we actually had to switch immune stimulants, and that was probably good for us.

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

And we also wanted to know: If we did a more remote immune stimulation, what would happen in that case? There were other reasons to suspect this might be worth looking at.

But we used a foot pad injection of a low level of Freund's complete adjuvant, and then evaluated cleft palates.

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

Interferon-gamma injection reduced to about 46 percent the cleft palate incidence. And then, of those clefts that we had, only 45 percent, rather than 85, were what we considered the more severe, or the wide cleft palates. So there's a change in two directions here.

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

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

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

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

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

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

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

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

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

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

This is cyclophosphamide that produces craniofacial or limb defects.

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

Diabetes mellitus, I've mentioned.

Methyl nitrous urea.

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

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

This was an interesting experiment for another reason. This is the first case where we saw a defect that was apparently caused by the immune stimulation. Mice that were exposed to sodium valproate and also Freund's complete adjuvant, a significant number of these mice were born without tails. That's not typical for sodium valproate. That's not a defect associated with this drug. It is very rare in the ICR mouse model we use, an anuria defect. So we're presuming--We've only done this experiment once, actually. This is, I think, the only one up here that's non-replicated. But we did see an increase in anuria, or tail-less mice, in this case, which was kind of interesting. X-rays, again, here, also.

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

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

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

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

The earlier report in 1990 suggested that the mechanism might involve activated immune cells that cross the placenta and find and eliminate pre-teratogenic cells. And they actually presented what I would say is limited data. And they readily admitted that this might not be the operating mechanism. It wasn't oversold by any means, but simply suggested.

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

But beyond that, for some of these chemical agents--and dioxin is a good example--the defect, the cleft palate defect in this case, is associated with a failure of apoptosis of cells lining the palatal shelves. This is an event required prior to proliferation of the underlying mesodermal cells that will then cause closure of the palate.

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

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

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

[Laughter.]

DR. HOLLADAY: We did perform a cell tracking study to see if we could track cells across the placenta, activated immune cells from mother to fetus, using a probe, the chloromethyl dichlorofluorocene diacetase--quite bright on flow cytometer. And the gist of that site was, we couldn't do it.

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

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

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

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

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

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

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

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

This is kind of a novel thing. It struck me when we saw that, that maternal immune manipulation is altering expression of very critical cell cycle controlling genes in the fetus. So we thought about the fetus for so long as a genetically pre-programmed entity that derives nutrition from the maternal organism, but other than that largely directs its own development. And these data would suggest that maternal influences might be more than we've thought.

And the immune system in this case is exerting an influence on gene expression in the fetus--protein, KNAC, alpha gene. And the protein products of this gene can influence expression of both BCL2 and P53. We evaluated that. And you can see that urethane drove that expression level down. And immune stimulation with one of these, Interferon-gamma, increased it.

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

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

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

This is available in a software package--it's on the Web--from the University of Pennsylvania. I'm seeing a little bit more of this type of analysis, as we all fight with how do we evaluate expression of multiple genes simultaneously.

With Freund's complete adjuvant injection, you'll see that the coordinate gene expression--these yellow squares--is shifted down, so it's normalized along this PC3 axis.

With Interferon-gamma injection, it's shifted further, so it's beyond normal along PC3, and closer to normal along PC1.

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

So we've been developing hypotheses as to what is occurring, what underlying effects are responsible for immune protection against birth defects. One of our hypotheses now is that immune stimulation is acting, at least in part, to restore dysregulated apoptosis.

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

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

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

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

So what I'm trying to do is collect enough data that it becomes compelling. Again, our gene expression data showed that the teratogen caused a shift in the BCL2-to-P53 ratio, that would lead us to predict increased apoptosis is involved in that defect. Immune stimulation with either of two stimulants shifted this ratio back towards BCL2, and that's a shift we would predict would be in favor of proliferation over apoptosis.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

[Applause.]

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

But you had a question for Dr. Holladay?

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

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

DR. HOLLADAY: Those are both very good questions. The immune stimulation timing is important. For instance, with diabetes, if we stimulate after development of hyperglycemia, we can't block the defect. Stimulation has to occur at a time of normal glycemia.

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

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

PARTICIPANT [In Audience]: Thalidomide [inaudible].

DR. HOLLADAY: Thalidomide, okay. Well, we've not used thalidomide. But it raises another interesting issue, in that so many teratogens are also immunotoxic, and I'm an immunotoxicologist. And I hadn't really made this connection before, but all of the teratogens we've worked with here, the chemical teratogens, are also immunotoxic. The dioxin is a wonderful example.

And it raises the question, if maternal immune stimulation reduces teratogenesis, how about the flip side of that? Is maternal immune suppression in itself an event that increases risk of teratogenesis? And thalidomide would fit well into that picture. And I don't know the answer to that. But to me, it's become an interesting question.

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

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

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

DEVELOPMENTAL IMMUNOTOXICOLOGY

PRESENTER: RALPH SMIALOWICZ, PH.D.,

U.S. ENVIRONMENTAL PROTECTION AGENCY

DR. SMIALOWICZ: Thank you, Marion.

This is going to be quite a divergence from the discussions and presentations that have occurred thus far. The

Environmental Protection Agency is not interested in vaccines. It's interested in environmental chemicals that humans are exposed to. And consequently, the work that we do deals with that.

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

[Tape Change.]

2B DR. SMIALOWICZ: Now, let me get all the equipment together here and start.

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

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

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

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

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

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

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

This is the hematopoietic scheme for the human. And I'm not going to go through that, since it was covered earlier. I just want to indicate the big difference, as was indicated earlier by Dr. Insel, about the fact that the rat and mouse, the rodent species, are much less developed at birth than is the human for immune system responses.

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

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

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

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

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

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

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

When we do immunotoxicity testing, we have a paradigm that we employ to look at different aspects of immune function. And this is a litany of the types of tests that will apply: Spleen and thymus weights, cellularity, body weights. Ex vivo types of tests include the splenic natural killer cell activity; assays of splenic lymphoproliferate responses to mitogens; in the rat, salmonella type for murine. Antigen is an LPS, like in the mouse, but it doesn't respond as well as the mouse does to LPS-induced responses.

The mixed leukocyte reaction: Here we have a problem again with the rat versus the mouse, in that the spleen cell for some reason has what we call "suppressor type cells" that don't give rise to a very good or robust mixed leukocyte reaction. And so we use lymph node cells in the rat model for that particular assay.

We use flow cytometric analysis. Depending on what in vivo and/or ex vivo type test that we do, we'll look at spleen, thymus, and/or lymph nodes.

We look at cytokine profiles, to try to see if there are any changes in the profiles. We've used the ribonuclease protection assay, which is one where you have several different cytokines that are expressed or can be identified on gels. And we purchase those for the rats. Some of that is strictly TH1, versus TH2 type cytokine profiles.

In vivo tests, which really are turning out to be the most sensitive tests to determine if a chemical is a developmental immunotoxicant--and actually, an immunotoxicant per se: The primary and secondary antibody response to sheep erythrocytes. You can do that with a platforming cell assay or the ELISA assay. And we've also used KLH.

Delayed-type hypersensitivity response: We've used bovine serum albumin, and KLH, using the foot pad swelling test. And you can also use those animals to measure immunoglobulin responses to that antigen.

We've used the contact hypersensitivity response to DNFB, dinitrofluorobenzene [ph]; looked at penis swelling, ear swelling tests in the rat. And have also used host-resistance models; one including the T.spiralis infectivity model.

So let me just give you--Okay, this is a time line for immune responses to sheep red blood cells, that was published in 1985 by Kimura et al, demonstrating when you could really start to pick up immune function in these animals as measured by the platforming cell assay, with sheep red blood cells as an antigen.

And as you can see, you can get demonstrable effects and responses here at as early as 20 days postnatal. The peak response occurs at postnatal day five--45 here.

This also is the same type of pattern that you see with the T-independent antigens, T-independent-1 and T-independent-2 type antigens, the TNF, LPS types.

So you can measure in the rat at about weaning an immune response to these different types of antigens. If you go down any earlier than that, you're going to have a lot of trouble picking up anything.

These are the chemicals that we've looked at: di-N-octyltin dichloride, and tributyltin oxide. Di-N-octyltin is used as a stabilizer in the production of polyvinylchloride materials. Tributyltin oxide is a mulluscocide and a fungicide, and is used in a lot of paints and especially as an anti-foulant on ships and boats.

Good old TCDD, one of the most studied of all immunotoxicants.

Methoxychlor, which is a pesticide--one of only four organochlorine pesticides that is allowable in the United States, based on EPA's, basically, elimination of many organochlorine type pesticides.

And then, heptachlor, which is another organochlorine, which has been banned for about 25 years now.

So we have looked at these five different chemicals, and tried to determine: Could we find an effect on the development of the immune system? If we find an effect in the immune system, is it a dose-related response that we see when we look at the immune functional end points?

We also are interested in knowing if this exposure during the development of the immune system is more severe than if one were to do the same dosing regimen in an adult animal, to determine if there is a difference in the sensitivity there.

Another consideration here is the pharmacodynamics, particularly metabolism of the chemical and its distribution. And I'll give you examples of that as we go through these slides.

The first group of studies we did were organotins. Basically, what we did, originally we looked at the prenatal exposure, and found that there were no effects whatsoever on the immune system of these rats.

We then decided to go and look at the newborn animal, starting on gestation day three, through 24; dosing those animals over a period of time, for a total of ten doses, with either the di-N-octyltin dichloride or TBTO.

I want to point out here that there is discussion about dosing or exposure of vaccines to animals. You can gavage a three-day-old rat. You have to be good at it, but you can do it.

In any event, then we looked at this time line, looked at the variety of immune function assays, and I'll show you those right now. This is four weeks, actually, four weeks of age. And this is just basically four days after the last exposure of these pups to the chemical. This is DOTC. You see that we get dose-related suppression of all the mitogen-stimulated responses, the T cell mitogen responses and the B cell mitogen response. So this is just four days after the last exposure.

We still see this suppression up to seven weeks. Okay? So now we're talking three weeks post last exposure. So these animals still have a suppressed response, as measured by the mitogen responses here.

After that, we checked them again at ten weeks, and they had returned to normal. So this is not a persistent suppression, but it's a somewhat long-lived suppression, at least for these functional end points.

With TBTO, we found effects on the NK cell activity. Here we used two different targets: the yak [ph], which is used primarily--It's a mouse lymphoma; and the WFU, which is a rat lymphoma. And basically found effects at four weeks, which is three days after the last exposure. However, subsequent to this, there are no effects on the NK activity.

These are the mitogen responses. And we also included a mixed leukocyte reaction here. This is three days--four days after the last exposure. Basically, another dose-dependent type of response and suppression of mitogen response and the mixed leukocyte response. We went up to ten weeks, and we still saw suppression here; only at the high dose, however. And again, this is a bit more long-lived an effect than with the di-N-octyltin dichloride.

So just as a summary--I'm not going to go through this, but I just want to point out that we did expose adult animals. These are all done in male animals, by the way. We did do the same dosing regimen with the adult males, and did the different tests, and found no effects whatsoever at any of the doses that we used. So obviously, the developing immune system of the rat, exposed to either of these two organic tints, caused "immunosuppression."

The next group of slides that I'll show you are TCDD. We're looking at a single exposure to TCDD, or dioxin, on gestation day 14, and how that affects the immune system. TCDD is a known immunotoxicant, as I said. There's a lot of work that's been done with it--and actually, work prior to what we did here--by Vos and Faith and Jack Moore, that demonstrated that this is a developmental immunotoxicant. And we decided to look at it a little bit more closely. So basically, we look at--This is a time line, basically. We dosed the animals, the pregnant animals, on gestation day 14. This is by gavage.

We looked at phenotype. We know that there are changes in T cell populations, a block between the double-positive CD4/CD8 to the--double-negative to the double-positive CD4/CD8 in these animals.

And then we looked at a host of different immune function assays. And what we found was that the DTH response was one which caused effects up to 19 months of age. And let me just show you those data.

Okay, what we did, this is a cross-fostering study, talking about dynamics, pharmacokinetics, and metabolism, and that sort of thing; although this is not a metabolized chemical. With the control we have no effect. This is a dose of one microgram-per-kilogram on gestation 14. Placental: There is placental transport, but we don't have a change in the response. Lactational exposure only: We know that TCDD is found in the mother's milk. No effect. But when we look at the placental and lactational, we get a suppression.

This is animals that we did dose response here, looking at how low could we go to see an effect on the developing immune system. This is the DTH response, I'm sorry. The previous slide is the same.

Basically, the DTH response was the most sensitive response, and so we focused on this. Basically, what happens is that at four age you see a dose-related decrease, but it's not significant. However, when you get out to 14 months--I'm sorry, 14 months of age--we had across-the-board suppression of the DTH response. We also looked at a higher dose, 3 microgram-per-kilogram. And this is the data that goes out to 19 months of age.

More recently, we've looked at the effect that TCDD given on gestation 14 has on the DNFB ear swelling response in the rat. And as you can see, at two months old, there is an effect, at 3 micrograms-per-kilogram; and again, at four, an effect.

The interesting thing, we did this with both BSA--The data I just showed you was with the BSA adjuvant. The other antigen that we use is KLH. And we found the same kind of effects with the KLH-sensitized animals.

This is data from Fan et al, 1996, in which they looked at the suppression of the DTH response to KLH in animals exposed to TCDD. It took a dose of 90 micrograms-per-kilogram to cause a decrease in that particular response. So we're talking about at least over a tenfold difference--a hundredfold difference--in the dosing where we're going to find an effect in a developing animal, versus an adult animal, using TCDD as the toxicant and the delayed-type hypersensitivity as a metric.

Okay. This is just a summary of this; again, highlight the work by Fan. And here is a computer here. This is the KLH adult study where the DTH took 90 micrograms-per-kilogram to suppress the response.

All right. This is a schematic of a group of studies that we did with the National Institute of Environmental Health Sciences primarily, orchestrated by Bob Chapin [ph], a developmental teratologist.

In the early '90s, the National Research Council, under the auspices of the National Academy of Sciences, wrote a document--and the title of that document was "Pesticides in the Diets of Infants and Children"--because of the concern for children being potentially more susceptible to exposure to different types of pesticides.

And so what we did was, we developed a dosing scheme and testing scheme, that is illustrated here. I know it's real busy, because it has not only the immunotox, which is here, but also the developmental tox, and repro-tox and neuro-tox. But let me just focus on this part here for the immunotox.

Basically, we did the dosing starting around gestation day 14, and in some cases on gestation day 12; dosed the dams; continued to dose the dam for the first week, so that the pups were exposed via lactation. And then after that, we directly dosed the kids. And the reason why we dosed the kids, because this would be closer to what would be happening in young children.

And they're still getting it from the dams. The dams are no longer dosed, but they still have some of this whatever pesticide in the milk, if it is in the milk. And then, we stopped at six weeks of age; we wait two weeks; and then we look to see what happens.

We did five different pesticides. We did carbaryl: Found no effect there. We did tebuconosol [ph], which is a fungicide: No effect there. We did chlorporophoz [ph] [inaudible]: No effect there. However, we did effects in methoxychlor and in heptachlor, and let me just show you those data.

These are nine-week-old male pups that were assayed for their response to sheep red blood cells. And you can see that there was a dose-related decrease in the antibody response to sheep red blood cells at the very lowest dose and the mid dose here.

We didn't have any other animals that we could use to look further into other immune function end points, unfortunately. So that had to wait for the work with heptachlor.

Now, the heptachlor work is interesting in that heptachlor is no longer used as a pesticide. It's banned in the United States. However, there was an incident in Hawaii in the late '70s and early '80s where heptachlor was used to control mealy bug on the pineapple plants. And as is the case in a lot of agricultural endeavors, the pineapple plantation owners were interested in using every part of that pineapple plant.

Consequently, what they did was they took the leaves from the pineapple and basically shredded them up, and added it to what they call "green chop," which was fed to dairy cows in Oahu. It was only in Oahu. And what happened was that the cows' milk was contaminated with heptachlor, obviously. The doses that we chose here were based on a low dose of 30 micrograms heptachlor per kilogram per day, in dosing these animals. The reason being that that dosage was within the 95th percentile of the amount of heptachlor epoxide--which is the major metabolite of heptachlor--that 95th percentile of what was found in mothers' milk on Oahu. So these data are relevant, from that standpoint, in this heptachlor fiasco, if you would.

This is just some pharmacokinetic metabolism information. Basically, the blood, thymus, and spleen had about pretty much the same levels. Obviously, the fat had a lot more, because this is a lipophilic, organochlorine compound so you have a lot in the fat. And because it's in the fat it's of concern because if these animals were not exposed post-natally, as the pups were being breast fed they would continue to be getting that heptachlor epoxide.

What we found here, this is the antibody response to sheep red blood cells in eight-week-old mice. This is two weeks after the last exposure, and we see a nice dose-dependent decrease at all doses that we examined.

And then, 26 weeks later, now we're talking about basically 20 weeks after the fact. The IgG response: The same antigen was reduced, as one might expect; but not necessarily expect it to be as "persistent" as it apparently was.

We also looked at the DNFB response. And I must mention, for all of these--for the TCDD work and for this work with the pesticides--we looked at both males and females. It's an important consideration, given that what we're finding is that males seem to be more susceptible than females. Why, I don't know.

But basically, this demonstrates the suppression of the DNFB response, ear pinna swelling, in the males that were exposed to the lowest dose, to the highest dose.

Again, this is just a summary of what I just showed you. But I want to point out that we looked at the dams. Now these are the females, so they're not going to be as sensitive as the males. But we looked at these females, and we saw no effects after weaning.

What we're doing now is we're trying to dissect the developmental sequence, those periods of developmental susceptibility; dosing the animals during those periods to find out if there is in fact one or two, or maybe many, critical periods of development that would be affected by exposure to this particular pesticide.

Now I want to talk about something that FDA is interested in, and that's drugs. It has nothing to do with vaccines. But this is work from three different laboratories.

The first one is diazepam: Work by Schlumpf et al; did a lot of work with this; used the rat. And in their studies they used both males and females; no real distinction between males versus females.

But nonetheless, a subcutaneous injection on gestation day 14 or 20--of the dam, obviously--at 1.25 milligrams diazepam per kilogram. They demonstrated decrease in T cell responses, ConA, and mixed leukocyte reaction; decrease in the plaque forming cell assay to sheep red blood cells at eight weeks; alterations in the ability of spleen cells, macrophages, and thymocytes to produce different types of cytokines--the TNF-alpha, IL1, IL2, 6. And this is in four- to six-week-old animals.

And finally, kind of the real acid test for an immunosuppressant is what happens when you challenge it with an infectious agent. And they found suppression of the T.spiralis infection in eight-week-old animals.

I apologize for all these computers and signs I don't recognize. Must be a different version of Power Point, of something.

Dexamethasone: A steroid. Bakker did a lot of work with this. He has several papers, but this paper in 2000 from the JI indicates that there are increased signs of guinea pig myeloid-based protein/complete Freund's adjuvant induced neurological tail tonus and paralysis and hind limbs of these animals. So it's somewhat of an autoimmune type reaction that was demonstrated with the dexamethasone.

Also, there were changes: Down regulation of certain types of cytokines, LPS-stimulated cells and ConA-stimulated cells; decreases in a variety of different cytokines. And also, an increase in spleen production of TNF--and I believe this is gamma, Interferon-gamma, and IL2, at nine weeks old.

So what you have here is kind of a mixed bag of both: an autoimmune type exacerbation of a response to the protein, and some indications for immunosuppression as well.

Acyclovir work, from Stahlmann's lab, using 10 milligrams per kilogram; and this is gestation day ten; subcutaneous injection, either once or three times. And basically, changes in body weight, so there's some toxicity, overt toxicity obviously, associated with this exposure; but decreases in thymus weight in males and in females.

Again, the test with the T.spiralis, trichnospiralis [ph], looking at decrease in the infection, protection against this particular parasite, as well as decreases in the antibody response to that parasite.

Now, finally I come to the human situation. And these are epistudies that deal primarily with organochlorine chemicals.

In Canada, Dewally did work with Inuit Indians in Quebec Province. These are subsistence hunters and fishers, and they are eating wildlife and fish that are highly contaminated, with a variety of PCBs in particular.

And so what they did was they looked at possible problems in the young children born to the mothers of this particular group--this tribe, I guess you would call it.

And what they did was, they were able to associate levels of DDE, hexachlorobenzene, dieldrin, as measured by the amount of these different chemicals in breast milk, and associate that with an increased risk in otitis media.

And then also, they found that that also included the hexachlorobenzene and dieldrin. And this is in one-year-old Inuit newborns. And the population that they studied was 171. So what that says is that these particular children are suffering from otitis media more so than children that are not--based on the levels of these different chemicals in the mothers' milk.

PCBs and TCDDs work was done by Weisglas-Kuperus. This is from The Netherlands, work from The Netherlands. This is a cohort that's been studied for many years now. In the last iteration--It's not really the last, but in 2000--it was published.

Maternal cord blood and plasma and milk, served to the surrogate for the pre- and post-natal exposure to these organochlorine chemicals. They found an association with exposure to both of these types of chemicals, with a decreased antibody response to mumps and measles; again, an increase in otitis media and chicken pox; and then a decreased prevalence of allergy in 42-month-old animals--children, sorry, 42-month-old children.

This change, this decreased prevalence of allergy, may have something to do with a TH2/TH1 shift. They haven't examined that, but that may be what's underlying this decrease in allergy.

Finally, work by Karmaus--and this is from Germany--looking again at PCBs, DDEs, and hexachlorobenzene: They're looking at whole blood levels of these chemicals in the children that were examined. And the children were eight-year-old children, 340.

And again, what we see is another predilection to increased risk of otitis media. In this case, unlike for the TCDD-PCB work, asthma increased, as opposed to decreased prevalence of asthma or allergic type responses. But there was an increase in IgE. And that's in the seven- to eight-year-old children.

So what we have here are some examples of what can be associated with some of the effects that we see in the animals during the development of the immune system.

So what I'd like to do, to just summarize here: We've used the rat as a model, because the rat is the model primarily for toxicity testing. I think it's a sensitive species, rodent species, for identifying developmental immunotoxicants following either pre- and/or postnatal exposure.

The immune function that we looked at--innate and specific--can be successfully assessed from pre-puberty throughout life.

Alterations initiated during immune system development in the rat may occur at lower chemical doses than those required in the adult.

With certain chemicals--and here we're talking pretty much about the organochlorines and diazepam--it appears that males are more profoundly affected, which may be linked to perturbations in the endocrine-immune network.

Selection of the immune developmental periods for chemical exposure if possible should be based on the pharmacokinetics of the chemical; as I showed with the trans-placental and lactational exposure to TCDD, versus what happened with the organochlorines where it's not passed either via the placenta nor the milk of the dam to the pups.

And from our standpoint, I think it's important--These are all screening now; this is not trying to get to the bottom line of how is this all happening. But for screening purposes, I would recommend that dosing encompass the in utero period, lactational, and pre-pubertal periods of development; basically, loading the deck, if you would, to try to identify potential immunotoxicants, from the standpoint of environmental chemicals.

Thank you.

[Applause.]

DR. SMIALOWICZ: Any questions? Okay. Nobody is coming up for questions, so I guess we're going to go eat.

Everybody's hungry, I guess.

[Pause.]

DR. SMIALOWICZ: Okay. Thank you.

[Whereupon, the workshop recessed for lunch, to reconvene at 1:15 p.m., that same day.

A F T E R N O O N   S E S S I O N

3A DR. SERABIAN: There's going to be sort of a modification in the afternoon schedule as we have it. Basically, what should have been this morning we're going to start with this afternoon, which is topic one, "Study Design." Dr. Mildred Christian is going to give a short presentation. Then we're going to have a question-and-answer session similar to yesterday.

Then we'll go into topic four--because we feel that with those two topics, there's more of an overlap with those two than with the others--which is "Animal Models." And Dr. Barrow will again give a small presentation. Then we'll follow that with some question-and-answer session.

And then, approximately around three, we will end; we'll have a short break. And then we'll start after that with topics two and three; because again, those two, immunological and developmental endpoints, pretty much--There's a bit of overlap there, also. So we thought that was the best way to organize it. Okay.

And let me introduce myself. That might help. My name is Mercedes Serabian. Right now I am with the Office of Cellular Tissue and Gene Therapies, in Center for Biologics.

I just want to reiterate what Marion had stressed this morning. The questions that she put up briefly in her talk we're going to put up also during these sessions. And the questions do have a bit of overlap, but that's I think important, because it just shows that basically all the issues and topics that we have have quite a bit of overlap and need to be evaluated.

One big thing, though, is that even when they do overlap we're going to try to keep the session moving and the topics moving as much as we can, just to keep the afternoon moving along.

I just want to stress that, again, the ultimate goal of today's session is to present the guidance document, as was done, and the questions that both we and industry have had at this point; and to try to come to some type of consensus as to the questions and the revisions that we think need to be made to this document. And I think that's really crucial. And it is crucial for you all, as you are the manufacturers as well as the companies that test these agents. Okay.

Let me introduce the first speaker, then, which is Dr. Mildred Christian. Dr. Christian obtained her Ph.D. from Thomas Jefferson University, in developmental anatomy, and has been active in regulatory toxicology for more than 35 years.

After 14 years as a teratologist/toxicologist with McNeil [ph] Labs, which is a J&J subsidiary, she founded Argus Research Labs in 1979, Argus International in 1980, and the Center for Photobiology at Argus in 1989; at each of which she served as chairman and president.

She merged two of these organizations with TSI Corporation in 1991, becoming vice president of the TSI in vivo testing group of five CROs. Beginning with Genzyme [ph] Transgenics' acquisition of TSI in 1996, she has served as executive director of science and compliance for GTC's Primedica [ph] Corporation, after the purchase of Primedica by Charles River, until November 2002.

In this position she was responsible for scientific integrity and regulatory compliance for the CRL-DDS laboratories, coordinating the product management across the labs, and for reviewing protocols and reports generated by Argus Research.

Mildred has been personally involved in the evaluation and submission of over 1,200 developmental, reproductive, and general tox evaluations, interacting with more than 350 pharmaceutical, chemical, and consortium organizations supporting these activities.

She has also developed more than 1,000 position papers for chemical and pharmaceutical companies, the FDA, the EPA, the Office of Technology Assessment, and the OECD.

She has also been involved in the ICH repro-tox guidance documents, the "red book" document, and many, many other numerous documents that I don't have time to present at this point.

Dr. Christian.

[Applause.]

STUDY DESIGN

PRESENTER: MILDRED CHRISTIAN, PH.D.

EXEC. DIR., RESEARCH, ARGUS RESEARCH LABS

DR. CHRISTIAN: I will make a statement that sounds like I'm with the government now. These are my own opinions that will be presented, and not those of anyone else. The designs that will be described are those which we used in studies over the years, and they represent to some extent the development of the procedures in testing for these types of compounds.

The basics are that when one does these types of studies, as mentioned yesterday, they of course are performed in conformance with GLPs. That's basic.

Then we're supposed to have them do the route and frequency of administration that is mimicking clinical use. Sometimes, very difficult.

Consider the pharmacokinetics: Well, that's perhaps relevant to the adjuvant, as we heard yesterday, but not necessarily to the active portion of the compound; the pharmacodynamics, though, certainly, of these vaccines. Bioavailability--this is something important; the volume that can be administered. And then, identify dose-response relationships, something we've heard may not be too important, or even relevant, with these types of compounds. The reason I say that--and these are the considerations as compared with the basics--is that we're going to look at only one species--theoretically, the relevant species--which we did a great deal of discussion about, and will do some more later, as to what is relevant.

Clinical use: The clinical use is really that we are, at least in theory, addressing the immune response; which is quite different from the classic developmental toxicity study in which one would address the response to a drug or to a chemical.

And then, we are also looking at the potential toxicity of at least two components; one being the vaccine itself, and the other, the response to the vaccine with an adjuvant, and possibly of the adjuvant alone.

When we were developing the ICH guidelines, this is what we came up with. Now, these are the segments. And when you see reference to the ICH guidelines for reproduction and development, what is important--and one of the reasons there's some confusing nomenclature perhaps used--is that reproduction is the whole cycle. And it starts with reproduction, conception, and you go all the way through, and end up with maturity, the next generation, and sometimes go into senescence. And what we said for the ICH guideline was that we were to look at each segment.

Now, what is come up with for these types of testing was when the initial thought--And this was really something that Joy Cabanero [ph] and I worked with many, many years ago. The initial thought was--because no testing at that time of repro-tox--Would there be any effect of the immune response on development? And would that possibly cause the most expected changes in the endpoints: abortion, death of the conceptus, malformation, reduced fetal body weight?

So we were at that time thinking strictly in terms of the type of developmental toxicity that is usually evaluated in a developmental toxicity study, which ends at C-section.

Do you address function? No, because you don't look. They're dead. Do you address immune response? That wasn't normally done. But remember, what we usually did was we had to dose every single day of gestation, because every day is a moving target in the developing conceptus.

And so the normal developmental toxicity study starts about implantation; goes through embryogenesis, with exposure there, and that being the period most likely to result in malformation. After palate closure, during the fetal period, that's the period of growth. And generally, these two "C" and "D" sections, as you heard earlier, are the intervals that one is concerned about in a developmental toxicity study.

However, you've also heard that we should do boosters; we should do it at the time of peak response. And that results actually in having a study that starts pre-conception. And so we do do some evaluation of fertility already in the design, if we do the booster shots.

And to look through to weaning has been suggested, and that would certainly be some postnatal evaluation; although not necessarily, as I'll show you, sufficiently long to see if we had immune effects out late in life.

This is just a summary of some rather large points, to show that the human and the mouse, at least, are not the same. And we've gone over that several times. But I think what is important here is, if we are attempting to maximize response in the rodent species, it's really in the fetal and postnatal period; and it's in the first and second trimester in humans.

And this is a repeat of that showing in a mouse or a rat, with the maturation with immunocompetence going on one year; 30 days postnatal. Immune memory, going up to 18 years in humans; mouse or rat, 30 to 60 days postnatal. So we have different time points when the targeted tissues might be sensitive.

Now, what is the response of species, and when is the maximum response? If we look and take the concept that the maximum immune response should be present during the most sensitive period of gestation, classically that's usually considered the first trimester for morphologic changes; and need to initiate treatment before if we need a booster.

But we also have to remember we're going to give several injections. And ideally, we'll have to have information obtained about when we need to give those injections from at least non-pregnant animals, so that we can compare them with pregnant animals and see if pregnancy itself is something we need to be concerned about. That is generally not done.

Now, we all know certainly there are several components for vaccines. We should know the general toxicity of each of the components. And for the adjuvant, I think at the very least there should be an arm in the developmental tox study, if it is a new or unusual adjuvant. I'll show you what I mean by that, and why.

We have heard that the most common dose tested is one times the human dose. And in the studies which I'm going to show you, they were generally done for NIDA. And there was a series of them that were done based on when the maximum immune response would be reached.

There are also some that are proprietary compounds, and they were similarly either studied ahead of time, to find out when the maximum response would be present, or dosed sequentially with different sets of animals, so that that could be evaluated post-testing. And then one could look at when the maximum immune response was present, and identify which group was considered the most relevant for testing and evaluation.

It has to be remembered that sometimes the doses are limited by local toxicity. And that's very important in developmental toxicity because of the secondary effects of local toxicity. We know that if we were doing a dermal study and we caused remarkable irritation to the dam, there are certain things we would expect to happen. We'd have stress reactions that would result in secondary effects in the fetuses. Most likely, we would see such things as extra ribs; we might see some reduction of fetal body weight; we might expect to see some increase in resorption. We also know that if we're dosing before implantation and we have stress reactions and a boostered immune response, we may get a lower incidence of implantation. And for that reason, when we're doing artificial insemination in rabbits, or natural mating, with prior treatment, we add more animals to the study, simply to ensure that we have sufficient numbers that become pregnant for evaluation. Often, more than one dose in the series of studies I'm going to show you that we performed; but seldom is there even an attempt to show a classic dose response. And that's appropriate.

How many doses are generally tested? That's certainly on a case-by-case basis. And it would be dependent not only on the onset of the response, but also on how long it lasts, the pharmacodynamics of the compound.

And then, of course, the effect of boosters. Whether it increases the response, maintains it, or whether it's going up and down during that whole interval, is important.

Now, the developmental tox endpoints to look at, I would think, certainly would be, at a minimum, the classic ones, but would go through birth. Why? Because the immune system, if that is one of the target organs, isn't going to be even partially developed to an appropriate extent until postnatally.

This is just my own impression: Unless there is a particular need, I would not add in crown-rump length because it's a very insensitive parameter, in that it's highly variable, particularly in rabbit species. It's a little bit better in small rodents.

Organ weights: I put in I don't know that they would be necessary. They are highly variable when there is a selected number--and that number, if there's only one or two per litter that are taken. And of course, because it is a developmental tox study, the litter would be the representative unit.

And we found in our laboratory, unless we have at least three on average on the basis per litter for males and females, that the organ weights are not truly representative of the litters, and that statistical analyses are often misleading, both as false negatives and false positives. So I would recommend, if we're doing organ weights, to do at least three per sex per litter.

Antibody levels can be looked at for the mother, for the fetuses, and should be looked at for the pups. And this would answer the relative questions about: Is it present, and does it persist? I don't think doing the whole kinetics as an initial screen, in the absence of other effects, would be appropriate.

One thing that must be considered is not only the immune response, but is the potential for antibody transfer present? And that is dependent on the placenta. Exposure in the conceptus may not be the same as it is in humans.

And for that reason, we chose, when we were initially putting some of the study designs together, to use rabbits. Because placental transfer in the rabbit occurs, antibodies do cross, and it's much more similar to what happens in the human placenta than certainly the rodent. Or we've been asked sometimes to even do canine studies. And you must remember that certainly even a pig, it doesn't cross at all. And you'll be hearing more about species differences later.

Timing differences: Theoretically, we're to use the species--and this would be for any developmental tox study--the species with the best response, and with placental passage, and with the immune system most like humans.

And Paul will be talking a little bit later, but I'd just like to show you here. We do guinea pig occasionally, because of the longer time in utero and the comparable development of the CNS in the immune system to humans; not completely comparable, but both guinea pig and pig, closer.

Rabbit: Quite a bit postnatal. But it has two of the things: it mounts a good immune response, and you have placental passage.

Mouse: Maybe not. Most of the immunotox information there, but not quite as good a model.

We've done ferrets. One had a canine. Only responsive species.

Non-human primate: Perhaps. Very good, but very expensive, and limited in numbers; so not always the best model.

This is a summary of the study designs I'm going to show you. You'll notice that they were done either when their maximum response was present, or they were given at various times during gestation.

In all cases, they checked for placental passage. The rat was usually intramuscular. One test group generally at one times the human dose. Whenever there was a new or unusual adjuvant, it was tested as a separate arm. Most included shots that brought up injections pre-mating. And this gives us some indication of potential effects on the female fertility.

Some of them were followed postnatally. And the observations that were made there were generally for viability; growth; nursing activity, which in the rabbit is a very good measure of whether it has normal behavior or not. One has to remember, there is a certain number of rabbit mothers that don't like their babies, so you need some background as to what is the normal incidence of pup loss. And antibodies were looked at, both in the does and the pups.

When you're on maternal effects, something that should be remembered on a practical basis are daily observations. Because when one is injecting or administering a compound at weekly intervals, you want to follow the pattern of effect for developmental toxicity anyway, because the later days of the gestation may be those where the effects are. So if the injection is given on day one, the next day the mother may not eat, may lose weight; and you'll see a weight loss, and a weight gain. But if you only weigh weekly, you'll miss that. And if there are any effects on development, it wouldn't be seen. So even when the injections are given at weekly intervals, there should be daily body weights in your developmental toxicity study. And here what we did find was when there was daily treatment--And we have a study with daily treatment. Why?

Because every day of gestation the sensitivity of the animal changes towards the response that will occur, or potentially occur, in a conceptus. We found that there were effects on the dams that were not observed when there were fewer treatments. It's not remarkable; it's just something that one should be aware of.

We also found that the only studies in which we saw adverse effects on embryo fetal development were those in which the adjuvant arm showed similar effects.

This is a study design in mice. It was a developmental tox study, which meant treatment was limited to one week pre-mating, or gestation-six, or gestation-13. Why? Because that got at least one treatment during embryogenesis, one treatment that would occur over fetogenesis. And the dose was two times the human dose. We saw no effects in either the dams or the conceptuses.

This is a group of rabbit studies. In the range finding study, this is the longest one we had. Six weeks pre-insemination; three weeks pre-insemination. It had been already determined it was a three-week period to reach maximal response with a booster. And then during gestation, gestation days six, 12, and 18, with the vehicle alone, a high dose.

These animals were determined to be sero-positive at two weeks, and only those that were were continued on study. Doses at 1 and 2 "X." There were samples. They were worried about immune complexes. The kidneys were weighed. There were no effects on the dams or conceptuses.

This is a developmental tox study with daily dosing, seven to 19; a control; an adjuvant; a low and two high doses, one at the high dose of 20 times the human dose. This is one of the NIDA studies. It's a compound that has been used; it's a tetanus toxoid. It had been in use in humans. Two high doses, one which followed the seven and 19, and one which was seven and 12 and 18 during gestation. Here we had maternal toxicity in the daily dosing. No developmental toxicity in either daily or the weekly dosing during gestation.

Another developmental tox, beginning four weeks. And this is the weekly schedule, four, three, two, and one, pre-insemination. And then another dose on gestation day 18. These were samples taken of antibody levels. They were taken for the mother for baseline level before the first dose at two weeks, at four weeks, gestation days 18 and 19, from the fetuses at C-section. Antibody titres were present. No effects, or no adverse effects.

Another IM study: One week pre-; different schedule, two, six, and 13. In each case, these are based on predetermined information as to when the maximum responses were present. A placebo control, and three high doses, day two, day six, or day 13. And the mothers were bled, and antibody levels determined before the first dose and on GD-29, which is the day sacrificed, so that they could figure out if there was persistence or when the peak effect occurred.

This is another one: Two groups, IM. The difference is, you can tell the number of samples that were taken. This is the first set that would go postnatally. And there is another set that is taken at lactation day 21, when the animals were weaned. Four weeks, one week, sort of the standard after that--seven, 14, and 24. One times the human dose, which was 20 times the maximum human dose.

Fetuses at gestation 29; pups at lactation day 21; the mothers before each.

And the others are quite similar here, going the same way. But the important thing is we were making these determinations.

This is a ferret study, selected because that was the responsive species. A quite large study. Treatments were days three, six, 13, or 22. A vehicle and a high dose at one times the human, so there would be a vehicle and a high at day three, at day six, at day 13, and day 22. Samples were taken at termination on day 35.

What can we say about this? Well, most studies, the evaluations were limited to the immune response. The antibodies were studied in the dam, the conceptuses, and the pups, to determine either before or after what the peak levels were. Most looked at only one dose.

I think this is important. Most did not administer the test material or get a peak response in the animals during the period when the animal's immune system was developing. Because the purpose wasn't to look at the target of the immune system; but rather to see if we caused death, abortion, or malformation.

I believe that's very important. My personal opinion is, if we're going to consider the immune system the target, we'd better consider treatment postnatally of the mothers and seeing if the antibody comes across in the milk and if they continue to be exposed to it during the lactation period.

To date, no study we conducted with these types of vaccines looked at potential effects on the immune system. However, when we have used other types of vaccines and immunosuppressive agents, we have seen immunosuppressive effects that were not evident until after puberty.

And I think this is important. None compared the pregnant versus the non-pregnant animals. And if we are worried about the offspring, we should also be worried about the pregnant animal potentially being different from the non-pregnant animal. And the same applies, I would hope, to the pregnant woman versus a different potential sensitivity.

We look at potential effects on embryo-fetal development, but it's really only regarding the presence of antibody. The transfer and persistence can be addressed by looking at fetal levels and pup levels, and at least knowing whether it persists in the pups up until weaning. But if we want to look at immune function, the designs do have to be changed as FDA has suggested.

He says "No," but I think the EPA data also support that. Viability and body weight and growth are the best indicators today, 14 postnatal. After that, if it's a rat or a mouse they'll start eating material food; they're on their own; they're weaned; and the whole weight pattern and viability, there's a second dip in viability.

Dose response: The only dose response we saw were effects of adjuvant. I haven't showed you all the studies we've done, but just gave you some samples.

I think fetal tissue interactions are probably unnecessary, but possibly indicated on a case-by-case basis.

I don't think histopathology would be remarkably additive to the quality of this study, and would be only indicated if there were effects on organ to body weight ratios. And it must be remembered that, to have any value of them, we need at least three males and females per litter, and it should be evaluated on a litter basis.

So that probably gives you enough to think about. And I thank you for your time and the opportunity to show you some of these designs.

[Applause.]

DR. SERABIAN: Thanks, Millie.

We're just going to switch slightly, and I'm going to ask Dr. Barrow to give his presentation now. And then we'll combine those two topics. I think that's a much better use of time at this point.

Dr. Barrow studied in London, while working at the same time for the reproductive toxicology department at Beecham Pharmaceuticals. Over the last 19 years, he has worked for Cieros [ph] in Italy and France.

He is an active member of the American and European teratology societies, and is a frequent guest lecturer at faculties or facilities in Paris, Lyons, Strasbourg, and Toulouse.

Paul is presently director of toxicology at MDS Pharma Services Preclinical in Lyons.

ANIMAL MODELS

PRESENTER: PAUL BARROW

DIRECTOR OF TOXICOLOGY,

MDS PHARMA SERVICES

DR. BARROW: Thank you for that introduction. I'm very pleased to be here.

As a lead-in to the next discussion, I'd just like to give a rapid overview of some of the considerations that I consider important in species selection for developmental toxicity testing of vaccines. At the same time, I'll give a very rapid overview of some of the work that we've done at MDS on behalf of Aventis Pasteur of four new vaccines presently in development.

So we can start with the obvious question [Shown on Slide: "Which is the Best Model?"]. Every regulatory toxicologist hears this question at least twice a month; not only for vaccines, but for practically any therapeutic carrier you might think of.

And strangely enough, the reply is nearly always the same [Shown on Slide: "It's the Primate, Stupid!"]. Of course, the best model species is going to be the primate, for all developmental toxicity studies, or practically all.

It's worth remembering at this point that the very first regulatory guidelines were issued by the FDA back in 1966. And this was a direct response to the thalidomide tragedy. Thalidomide, as it turns out, is practically only teratogenic in primates, at least at human therapeutic doses.

However, even back then we decided--Well, that's the royal "we"; I was seven years old. Even back then it was decided that we would use rodents and rabbits for our routine developmental toxicity screen.

And the reasons for this are just as valid today as they were 40 years ago. There are just not enough primates in the world to supply our routine needs for routine developmental toxicity testing. And this situation is getting worse, not better; with practically all Western governments being very reluctant to license new primate breeding facilities on their soil.

To make matters worse, to get a valid developmental toxicity study in the primate we need to use relatively high group sizes. To start with, each monkey normally only has one fetus per pregnancy. And also, primates don't tend to reproduce well in the laboratory. They have a high abortion rate of around 15 to 20 percent. So in a typical primate study, we're lucky to obtain ten fetuses per treatment group to examine at the end of the study; as opposed to 200 more per group in a typical rodent study.

One other disadvantage of primates which is particularly pertinent to vaccines is their long life span. If we want to expose primates pre- and postnatally, and then look at the functioning of the adult immune system, we're going to have to wait four to five years. Now, I don't know many of you out there that have that sort of patience.

So what are the most likely alternatives? Perhaps we won't have the choice. Perhaps the vaccine is only immunogenic in the primate, in which case we can't justify other species.

The three most obvious alternatives are the rat, mouse, and rabbit. Although, after listening to Millie's presentation, I should have added the ferret and the guinea pig to that list. I haven't done that, because I haven't used them personally.

The rat is the most frequently used species in developmental toxicology. Also, we heard this morning that a lot of developmental immunotoxicity work has been done in the rat.

Having said that, there's no reason why we can't use the mouse. Anything we can do in the rat is also perfectly feasible in the mouse. The mouse also has the advantage of having the most studied immune system of any animal.

I should also have said that the rat is often the only species in which we do postnatal examinations for developmental toxicology studies with drugs.

The second most used species after the rat is, of course, the rabbit. But the rabbit is normally only used for prenatal toxicology. We don't normally do postnatal examinations in this species. As Millie said earlier, postnatal examinations are very difficult; although we can't always avoid it, as you'll see in a moment. And as we heard yesterday, a lot of immune tests are not valid, or simply not available, in the rabbit.

Here are some of the considerations that we bear in mind when choosing a species. Evidently we want to choose a species that does mount an immune response to our vaccine; bearing in mind, of course, there may be quantitative and qualitative differences in immune response between species. One point raised in the FDA draft is the timing and rate of maternal antibody transfer. I'll come back to that in a moment.

And also, we're going to want to be able to do both fetal examinations and postnatal examinations in our chosen species.

Coming back to maternal immunoglobulin transport, as we've heard, the big difference between primates and rodents is the timing of maternal antibody transfer to the offspring. In primates practically all maternal antibody transfer is before birth. As it turns out, according to the literature at least, this is also the case for the rabbit and the guinea pig.

In rodents, however, only about 10 percent of maternal immunoglobulin transfers before birth, with the other 90 percent transferring across in the milk or the colostrum. And other species, as it turns out, are even worse, with little or no maternal antibody transfer before birth.

Now, this is the strategy that we have used to test four new vaccines. We normally start off with preliminary studies to look at the maternal immune response in the pregnant animal, and also to look at the timing and rate of maternal immunoglobulin transport, in each of three species: the rat, the mouse, and the rabbit.

And on the basis of these results, we normally choose just one species, to go ahead and do the main developmental tox study. We normally hope to be able to use a rodent because, as I said, the postnatal examinations in the rabbit are very difficult, although we've not always been able to avoid this.

So in the preliminary study we start with groups of 12 female animals of each species--rat, mouse, and rabbit. I've gained some new characters here. I didn't make that choice of bullet point. I think these are probably the characters that were missing from Steve's presentation this morning.

We treat animals of all three species before mating, according to a predetermined vaccination schedule which is based on the known immune response in that animal, and also on the proposed vaccination schedule in humans. So in a typical study, we'll treat the animals two or three times before mating, at ten-day intervals.

After mating, we then give all the females a booster vaccination on day six of gestation. This serves not only to maintain high maternal antibody levels throughout the remainder of gestation, but also hopefully to expose the developing embryo to the actual components of the vaccine formulation.

Six females--that's half of the females of each species--are then sent to caesarean examination, where we take blood samples to look at fetal titres and maternal antibody titres.

The other six females of each group get another vaccination at the end of gestation; are then allowed to give birth. And we kill off the females and pups on day 11 post-partum.

Again, we take serum samples to look at antibody titres in the pups and mothers. The FDA suggests that we also do antibody analysis in milk. Unfortunately, we've not been able to do that so far, because of analytical difficulties. This is an example of the type of results we obtain in this preliminary study. The blue blocks are fetal antibody titres. The red blocks are antibody titres in the pups on postnatal day 11, and these are expressed as a percentage of maternal titres. This was with an HIV vaccine.

We see here in the rat, fetal titres didn't reach maternal antibody levels before birth. In the mouse however, we did get a good prenatal transport. So we were able to justify the use of the mouse with this particular vaccine. As expected, we also got a good prenatal transport in the rabbit. I would also note that in all three species we did get a good persistence of maternal antibody levels in the pups up to 11 days of age.

So for the four vaccines tested to date, we were able to justify the use of the mouse for two of these vaccines: the HIV vaccine, and the tetanus/diphtheria/whooping cough vaccine.

Unfortunately, in two of the cases, we had to resort to using the rabbit. In the case of the meningitis vaccine, this was because of poor or unpredictable immunogenicity in the pregnant animal, in the pregnant rodent. But in the case of the rabies vaccine, this was because of poor maternal immunoglobulin transport before birth.

We then go on and do the main study. We use the same vaccination schedule as in the preliminary study. Here we start with groups of 40 rodents, or 35 rabbits. One subgroup of animals goes to caesarean, and we perform all the routine teratology type examinations. The other subgroup is allowed to give birth, and we do all of our postnatal followup on the litters following birth.

This second generation is normally terminated at weaning. Although if we do see any indications of developmental toxicity--which we've not done so far--we will extend the study to cover a postnatal followup, possibly with behavioral examinations, probably adding immune assessments; and perhaps even mate the animals to look at their fertility.

I would just like to ask one question before finishing, concerning comparative development and maternal immunoglobulin transport. I wonder if we've not been a bit misled by this. I wonder if we've not been premature in rejecting the use of the rat.

As we have heard this morning, rodents are very immature at birth, by comparison with humans. For instance, the erythropoietic activity of the bone marrow is already well in place in humans at the time of birth, but continues to develop postnatally in rodents. But we have also heard, nevertheless, the ontogeny of the immune system is fairly comparable between mouse and, I assume, the rat and humans. My question is: Are high fetal antibody titres really necessary, given that the critical period of immune development in the rodent probably occurs postnatally? And as we've shown, we do get good maternal immunoglobulin titres during this period. So providing there is a postnatal followup, we might not need to ensure exposure of the fetus to antibodies in rodents.

I don't claim to have any conclusions; though I do hope to have some information to fill in this slide by the end of today. So I guess now we just have to put the hand into the hat, to see what we can pull out. Thank you.

[Applause.]

DR. SERABIAN: Okay. I think we have about an hour, roughly, maybe a little more, to go over the two topics.

[Tape Change.]

3B DR. GRUBER: My name is Marion Gruber. I'm with the Office of Vaccines.

MS. MILLER: Margaret Miller, FDA, Office of Women's Health.

DR. VERDIER: Francois Verdier, Aventis Pasteur.

DR. INSEL: Dick Insel, University of Rochester.

DR. HOLLADAY: Steve Holladay, Virginia Tech.

DR. SMIALOWICZ: Ralph Smialowicz, the Environmental Protection Agency.

DR. CHRISTIAN: Mildred Christian, Argus Research.

DR. VAN DER LAAN: Jan-Willem van der Laan, The Netherlands, Medicines Evaluation Board.

DR. BARROW: Paul Barrow, MDS Pharma Services.

DR. HASTINGS: Ken Hastings, Division of Special Pathogen and Immunologic Drug Products in CDER, FDA.

DR. SERABIAN: Okay. I think initially we'll start off with--You have the questions in the pamphlet that you got. We'll start off with the first question, just because it's a rather broad question. And please feel free, you know, with any additional questions, to go up to the microphone stands. So this is just to start us off. Okay?

The first one is: In addition to endpoints outlined in the ICHS5A document, what additional parameters should be evaluated; such as immunological parameters, histopath, and functional assessment? It's what parameters; i.e., if you think functional assessment, what do you mean by that?

DR. VAN DER LAAN: Should we reserve this question to the last round? In fact, it is the endpoints session.

DR. GRUBER: Yes, we can keep this rather flexible. And we will just leave this up there, and we'll just maybe screen through the questions, trying to get some answers to some of them. But perhaps we start off the discussion.

Or if somebody has questions regarding the two presentations that you just heard, then please come up to the microphone.

MS. HELPERIN [In Audience]: Yes, Jane Helperin [ph], ID Biomedical Corporation.

This is a question for Dr. Christian. I was wondering if you could give us a little more information on what compounds you were looking at in the studies you were discussing? And also, with regard to the different animal models used and the study designs you used, what the basis for that was? Such as, was there any background information or historical information which caused you to choose the designs you chose?

Because I think one of the reasons we're here is to try to figure out what rationale we should be using for study designs. So maybe you could give us a little more information on that?

DR. CHRISTIAN: Yes. With the exception of three of the compounds, they were all NIDA vaccines that were used either for--There was a flu, a tetanus, a hemolophius--Yes, there was an HIV, and an influenza.

And there was background data on each of those that told us the time for the booster shoots and how long it would take to get the maximum response.

All of those studies that were performed were performed for the purpose of evaluating whether they caused abortion or malformation, or affected fetal size in utero. None of them were done as functional assessments of postnatal development of the immune system, because that was not looked at as a target.

Rather, there were concerns whether immunization of pregnant women, particularly in Third World countries--if that would be a problem that would cause them potentially to have problems with morphologic development of their conceptuses.

And so they were designed with that in mind, and without a postnatal phase; other than in, I believe, six of them: evaluation of viability and persistence of the antibodies in the milk and in the pups.

PARTICIPANT [In Audience]: I have a question for Mildred or Steve or anyone who would like to answer it. But it seemed like some of you had looked at thymus-to-body-weight ratios. I always felt that was a very sensitive indicator for developmental immune changes. And did you look at that? And did you find it not to be the case? Or did you just not look at it?

DR. HOLLADAY: For all of the chemicals that I showed you, we looked at them and we really didn't see any effects on thymus-to-organ ratios, or spleen-to-body weight.

DR. CHRISTIAN: We didn't see any, either. But we did look at it in four of them.

DR. SMIALOWICZ: Well, Mike, you and I published a paper together in '96, EHP, evaluating fetal immune parameters and their sensitivity for indicators of developmental immunotoxicity. And of the indicators we found that were most sensitive, fetal thymic cellularity was among the sensitive ones in mouse models. When we correlated those data, they were more sensitive, or that was a more sensitive endpoint than fetal thymic markers, which occasionally didn't change when cellularity went down.

I contrast, cellularity of the fetal liver was a relatively poor marker of developmental immunotoxicant exposure. But marker expression in fetal liver was a pretty good indicator of developmental immunotoxicity.

So the summary of what I just said, according to our review in '96, is that fetal thymic hyper-cellularity is often a very sensitive indicator of developmental immunotoxicant exposure. It will, of course, depend on the chemical that is being evaluated. And fetal liver marker expression, again, is sometimes very sensitive.

I think DES and TCDD are beautiful examples. I suspect that the fetal liver progenitor cell may be the definitive sensitive cell for dioxin exposure. This is an exquisitely sensitive cell. So TDT positive cells in fetal liver in a mouse: pretty sensitive indicator.

DR. HOLLADAY: If I can make a little clarification here, we never looked at the fetus. We looked at animals that were at least--well, post-weaning. So we didn't see any effects there.

MR. STUMP [In Audience]: Don Stump [ph], World Research. I just wanted to ask the panel what their thoughts are on the designs as Dr. Christian and Dr. Barrow both talked about, immunizing before gestation and then also during various points during gestation.

Any thoughts on whether it's better to take the same group of animals and immunize them before breeding and through gestation; as opposed to taking subgroups where you have some animals that you only expose during gestation, some you only expose prior to gestation?

Because it's certainly differences you might see in terms of giving that vaccine to an animal that has not previously been challenged by the vaccine.

DR. CHRISTIAN: Yes, I think you have to do some range finding or pilot work first, to know that. And certainly, we did modifications based on when the responses were there. In some cases, we did multiple groups on separate days of gestation because the response--For instance, if we gave it on day six, it maxed about the middle of embryogenesis. And at other times, gave it pre, based on the onset of the effect.

I think it was most effective when given prior to gestation, and the booster given. And it probably had the least effect on the mother. What we were originally worried about when we started these studies--and that maybe was ten years ago now--was the potential effect of fever and its effect on each protein, and what would occur there. And we found that we didn't have any problems with that. That is different from some other types of vaccines. But with these therapeutic vaccines, it wasn't a problem.

DR. BARROW: I don't actually see the point in performing groups that are only vaccinated during gestation, unless we're trying to look at possible effects of other vaccine components other than the induced immune response. We have to treat them before mating in order to get a maintained immune response throughout gestation.

DR. VAN DER LAAN: May I comment also on that? I think it's pretty important the way that Mildred has presented the different days, the different periods during pregnancy. I think that that might give important information if you take your starting point from the clinical use of the vaccine.

If you give repeatedly a vaccine during pregnancy, that's never resembling the clinical approach. If a woman has been vaccinated before pregnancy, it's not clinical usage to do it again during pregnancy. So the most important problem is when the woman is pregnant, and then to be treated. And that might be important then, to know at which stage during pregnancy.

DR. GRUBER: Perhaps to further consider this point, I think what is apparent and what is important to really do in these studies is to administer priming doses prior to gestation. I think this has been becoming apparent from the discussions that we had today, and presentations. And it's also from discussions that we had when we looked, or when we designed developmental tox studies for these vaccines.

There is one point, or one question that I wanted to ask the experts. We have been recently considering, rather than giving multiple doses to the same group of animals during the period of organogenesis--let's say, between days six and 18--to really divide the animals into subgroups, and to dose certain groups at certain days of gestation only--for instance, to do it at day four, days six to ten--so that the animal is dosed then only once, or a given group is dosed only once. So of course they have been primed prior to gestation, or prior to conception. And then they receive one additional dose during gestation only. How do the experts feel about this?

And the reason why this is done is because we think that, especially if you look at vaccines targeted for adolescents and adults, many times you don't really give multiple doses to the human target population. So how do the experts feel about this type of design and schedule?

DR. CHRISTIAN: I'll start, and see if it can be controversial. I think the whole problem is the question. And if the question is inadvertent exposure of a woman who becomes pregnant, that's one question. If it is intended exposure, then the design is different. And there are vaccines with extended exposure during pregnancy.

When it's intended exposure, it should be started during the pregnancy, because that's the clinical use, and you know that the response will be developed during the pregnancy. And one might want to do that then with multiple groups during pregnancy, so that you could see the effect of how long it takes to mount the response during a pregnancy and when it's most effective. And that might be combined with an efficacy study, to evaluate at the same time both the effect on the pregnancy and the efficacy of the treatment.

If it's inadvertent exposure, as might occur when, let's say, we go to a country and just inoculate everyone--And many times certainly there are some countries where the people won't say they're pregnant. That would be against it. So they get inoculated. And now you have all different times of exposure. There it would be probably most appropriate to see the maximum response that can be maintained over the duration of the pregnancy. And a priming dose in that case would probably be appropriate, so that you could build up to the maximum response.

So it's really what question. And that, again, goes to the case-by-case use.

PARTICIPANT [In Audience]: Millie, are you talking priming, or frequency of dose? I guess I'm getting a little confused.

DR. CHRISTIAN: Well, actually, both. You'd want to do it before pregnancy, and then a booster shot to make sure--And you'd have to have some data, probably from non-pregnant animals, to know how to get to the maximum response.

Because the question would be: At the time of maximum response, what would be the outcome of that pregnancy?

PARTICIPANT [In Audience]: So basically, potentially a dose prime, and then a single administration at that time point? I'm just trying to understand. Versus several doses, you know, gestation days--

DR. CHRISTIAN: If the question would be--

PARTICIPANT [In Audience]: --six, ten, and 12, or something.

DR. CHRISTIAN: Yes. Would it affect implantation? You might want to do one before--

PARTICIPANT [In Audience]: Separately. Okay.

DR. CHRISTIAN: --mating; then one around the time of implantation; one at the time when peak morphologic development is ongoing; one when there's fetal development. And depending on the pattern of the response for a particular vaccine, the separation or even the need for additional doses would have to be determined. You know, if you can mount a response that's going to last the entire gestation, then you wouldn't give another shot.

DR. VERDIER: Just one remark regarding the difficulty to scale the vaccine administration, compared to the gestation period. The effect of the vaccine will not be immediate. I mean, you cannot say, "Okay, I will give the vaccine on day six of gestation to evaluate the potential adverse effect at this period of the gestation," because in fact the vaccine effect will last for several days, and will not start immediately after the administration.

That's why it's quite difficult to adjust the vaccine administration with the gestation schedule. And that's why I think we should say, okay, we start--Perhaps we should consider a very large period and say that, okay, we give the product on day six of gestation, in order to cover day six and perhaps the next ten following days.

Unless we want to evaluate the toxicity of one chemical constituent of the vaccine. But I think in this case, that's not the right method. If you want to evaluate the--

DR. CHRISTIAN: That's a different question.

DR. VERDIER: That's a different question. I mean, in this case we have to refer to the ICH5 guideline, and study the teratology of chemicals by normal way. But I think that's not the discussion now.

DR. CHRISTIAN: No. Maybe I was misunderstood. If one knows when the peak response is present, you might have to give it before mating so that for the duration of the most sensitive period, let's say, in a rat, essentially days six to 20, and possibly staying in maternal milk--And going over and being exposed that way. It might be fine to give it ahead of time, if you had that long a duration of response. If not, one might have to give an additional booster shot, or even two, before mating.

And that's why those designs--You notice there was one that had four pre-mating, and it started way out six weeks before mating, because it took that long to build up the maximum response.

DR. VAN DER LAAN: What do you mean with "the maximum response"? What's the most risk-full effect during pregnancy? Is that the existence of antibodies? Is that the transfer of antibodies through the placenta? Or is that the increase of cytokines, interferons, and all of those other elements?

I have the feeling that we should be aware of where we are talking about. Are we defining the maximum response as the antibody response, or other types of responses?

And that's also a question to Dr. Barrow. In his talk he indicates the selection of species based on the placental transfer of the antibodies.

DR. BARROW: Yes, that's a good question, to which I don't have an answer. Perhaps I could pass it over to another member of the panel.

[Laughter.]

DR. CHRISTIAN: Out of naiveness, like most of immunotoxicology, this is a rapidly evolving field. We don't have--Certainly, I don't have all of the answers.

But what I was talking about in terms of maximum response, what we were looking at was maximum levels of antibody production.

Of course, with the placenta we know that the permeability of the placenta, and the passage, and the way it goes across the placenta, change with gestation; with the placenta becoming more permeable as gestation continues.

So that, again, is changing with time.

And you're exceptionally correct with the cytokine production. We are concerned about that, because that would be what would induce a potential response that's secondary in the conceptus. However, whether or not we know what to measure certainly would be on a case-by-case basis, and modeled for that particular compound.

DR. HOLLADAY: I'm speaking from an immunotoxicologist's perspective. But clearly, there are data that different immunotoxicants have different windows of susceptibility prenatally. Chlordane is a good example; lead is another good example.

I think of immunosuppression typically in the work that I do. And in the case of this meeting, what I'm hearing so far, I'm not overly concerned about the effect of vaccines on a postnatal immunocompetence. My thoughts are more in line with, I suppose, exaggerated immune responses, hypersensitivity disorders, possibly autoimmunity.

And I think now about a paper recently that came out by Anser Ahmed [ph], who exposed animals to one low-level dose of diethylstilbestrol prenatally; carried these animals until they were geriatric. And from all parameters assessed, they appeared normal immunologically, until a secondary DES challenge was given. And at that time it was shown that their cytokine production profile was skewed in a direction that would lead one to predict they might be more prone to develop an autoimmune disorder.

I could almost see that type of thing happening with a prenatal maternal immune stimulation that skewed the fetal immune development such that it could be a very difficult thing to pick up, but in the right person at the right time with the right environmental exposures or combined exposures, we might see a phenomenon like this DES phenomenon. It's going to be difficult to test for and to show, however.

DR. CHRISTIAN: I think one of the things we must keep in mind is that these are screens. And as such, we're doing the best job we can do with our current level of knowledge. It's not really a research project that one is doing when doing the initial screening for potential effects.

But we are totally dependent on the research area for identifying what potential effects we should be looking for. And it's that combination then and development that will occur with time. So we can't see things as set in stone.

The reason I put out the original studies was, at that time what people were worried about was malformation. Now we're worried about functional alterations. But we don't truly have all of the ways of looking at it yet. We've seen it with immunosuppressants, with immunotoxicants. But if we use those tools for general screening, we may not be sufficiently expert to have relevant information right now. And perhaps some of those things even will not be relevant in the future, but that's the development of research. And we have to consider them. And I think that's part of what we're trying to do here. Should we add it as a part of the general screening pattern? I can tell you, with other compounds that are immunosuppressant we have seen, just as Ralph has seen, effects that don't occur until late postnatal, after puberty. And that's the first they're picked up, with increasing severity.

But it would be impractical for us to do lifetime studies, as well. So what we're trying to do is figure out what can we do on a practical basis in a species that, at least as much as we know, would mimic the human clinical situation in terms of response to the vaccine, and make sure that there is exposure of the conceptus at some interval that was developmentally similar to the human conceptus.

MR. RENEE [In Audience]: My name is Foulouse Renee [ph], from GSK Biologicals.

Maybe as a feedback to the FDA, the panel, and the audience, I could explain how we design our reproductive toxicity studies at GSK. We do prelim studies, where we test in more than one species immunogenicity. And we select the dose on the basis of the prelim study, as well as the species. Very often, it is the rat.

Then we have for the pivotal study, we have all of the animals which are pre-immunized 30 days before mating, and all of the animals which are immunized only during pregnancy. Now, we immunize besides day minus-30 all animals on day six, 11, 30, 50, of pregnancy. So we try to have the vaccine present during key moments of development of the embryo--fetus.

So we try to maximize the exposure to the formation. And we have good evidence for immune response at these days.

We go for caesarean section at the end of the pregnancy for half of the animals, and we go for half of the animals to day 21 or day 25 after birth.

Now, after birth we follow the classical parameters, and include also postnatal development, neural development, by assessing the acquirement of the flexes. And we believe that this is maximizing the exposure. We are not looking for optimum levels of individual antibodies. And this has been acceptable everywhere in the world till now.

MR. : I probably missed this, but did you also have at the--what was it?--28 days after birth, did you, besides neurological evaluation, did you have immune function evaluation?

MR. RENEE [In Audience]: What we do is we do the neurological assessment of the pups at day 21. And if there are effects seen, then we can prolong until day 25. Now of course, we follow body weight and other parameters after birth. And we take antibody samples at day four, when we cull the litters to standardize the litters. And we can compare antibody levels at day 21 or day 25. And this is a good indication of exposure to antibodies coming from the mother by the milk. Very often, we find higher levels at day 21 or day 25 of age than on day five, for instance.

MR.   : But you don't do any antigen challenge assay or anything like that?

MR. RENEE [In Audience]: No.

MR.   : And do you do any immunohistochemical analysis of the immune-related tissues at that point?

MR. RENEE [In Audience]: No.

MR.   : Okay.

PARTICIPANT [In Audience]: Could I ask either the panel or our colleague from SmithKline to frame this question about interval and timing of dosing?

You know, I see two different kinds of vaccines that you might want to do these studies for. One is the sort of vaccine that you might give only one time, like flu or tetanus, during pregnancy; versus something that I'm very concerned about, sexually-transmitted disease vaccines, where you might give vaccines on some schedule like zero-one-six, or zero-one-three-six months.

And there, the individuals who are participating in an IND trial, in addition to being at risk for sexually-transmitted diseases, may also be at risk for pregnancy. And so there you would be getting a very different kind of vaccine schedule than you would for tetanus or flu. And would that affect this timing and interval of dosing in these repro-tox studies?

DR. BARROW: Yes, I think we would have to design the study accordingly. But we can't actually get away from giving animals a pre-mating vaccination. Because gestation is so short in the animal, we need to give time for the maternal response to develop; which of course wouldn't be the case in the human.

MR. WYAN [In Audience]: Hi. This is Michael Wyan [ph], from 3M Biologics.

We have hundreds of vaccines, both used in humans and veterinary vaccines that have been given to a number of different animal species. And we also have human beings that are exposed to different infectious diseases while they're pregnant, naturally exposed to infectious diseases.

My first question is, are we concerned? Are we testing whether or not an immune response has deleterious effects on the fetus? It would seem that we have ample evidence that the normal physiologic immune response is certainly not a toxic reaction.

Now, we heard Mildred Christian say that even in some of these animal studies you could have an irritation on the skin that could result in effects on--I forget what it was, Mildred. Viability of the pups, or whatever. So we know that general systemic reactions, such as inflammatory reaction, could have that kind of effect. But I mean, is that a toxic reaction? So I guess my question is, first, are we testing that?

And secondly, it seemed, based upon some of the things we've heard yesterday, our biggest concern would be an immune response that would cross-react with specific tissues or specific antigens. It might be mimicry, or it might be some other mechanism. So my second question is, are there any examples where we think that fetal antigens would be different than adult antigens, and would pose a different toxic profile to the vaccine?

So I guess part of me thinks that if you could examine a vaccine for tissue cross-reactivity and for safety in tissues from an adult, what's different about the fetus that's going to make this somehow a different problem? Are there any examples of a vaccine that is safe in adults, but is unsafe in children?

DR. BARROW: I'm not saying it's unsafe. In fact, I think the opposite. One example we could use are the group B polysaccharide meningococcal vaccines, where the induced antibody has been shown to target polyciliated molecules, such as neural adhesion cell molecules, which have a different form in the fetus to the adult.

In the fetus, these molecules are polyciliated and are targeted by the antibodies. In the adult, the molecules have been deciliated, and are no longer targeted by those antibodies. So that could give lead to a completely different reaction in the developing animal than in the adult.

PARTICIPANT [In Audience]: But I'd just like to ask Paul with that--and that's correct--has anybody studied that in an animal model in which--Now, here is a great example of cross-reactivity. And we've heard all about all kinds of models for immunotoxicants and for immunosuppressants. But here's a vaccine, and when tested in an animal model, a non-human primate or another model, do the offspring develop any kind of neuronal injury?

DR. BARROW: No, they don't. At least, we've not found any adverse effects so far.

MR. FREES [In Audience]: Lou Frees [ph], ID Biomedical. We've heard a lot of discussion of the need for the conceptus to encounter optimal levels of antibody in the mother, potentially cellular immune responses in the mother; also, to be exposed to vaccine at particular critical time points during development, as opposed to merely the maternal immunologic response.

I think one thing that strikes me as very important, coming back to one of the things that Dr. Christian demonstrated, is that I will readily concede that it is possible, by pounding a pregnant animal with enough doses of vaccine, to achieve an exposure. All those exposures in one treatment group are capable of injuring a pregnant female animal, and thereby her conceptus.

So I would only advocate that we plan these trials very carefully with multiple treatment arms, not every one of which is going to answer every one of these questions.

My second point would be again coming back to Dr. Christian for a moment, and reiterating something I said yesterday. It is not clear to me how doing a trial of an adjuvant only is of necessity for a regulatory package for registration; since the adjuvant only will never be presented to man.

I can see how it's a vital tool to the sponsor in understanding their product. But where the practicality is difficult or the additional manipulations that have to be added to make an adjuvant-alone study possible--many induce toxicities of their own--why is it necessary, or even desirable, to have an adjuvant-alone component to a reproductive toxicity program?

Clearly, if your vaccine demonstrates it, then the onus is on the sponsor to sort out what component of the vaccine is producing it. But if the vaccine, as it will be presented to humans, is benign, what's the additional benefit of an adjuvant-alone package?

DR. CHRISTIAN: What I presented wasn't a full package of an adjuvant alone. Rather, it was a novel adjuvant, which the sponsor wanted to know if it was toxic in and of itself. And what we saw was that it was the adjuvant that was quite irritating and produced the responses in the dam. And as a result, they changed the formulation and got another adjuvant, and went to one that was more standardized.

I think that when there are novel adjuvants, though, particularly in terms of developmental toxicity, it's a very good idea to study that, just as you would a vehicle or a placebo in a general tox study. Because you want to know if that is affecting the development of the conceptus. And if nothing happens, well, that's fine.

But by having a single arm there at the maximum dose, it sort of gives you a quick way to find out if something should happen at your high dose, whether it is the adjuvant. Although you're quite right--and I think this was your point--that it is in combination possibly different than it is alone, as well.

MR. FREES [In Audience]: Yes, that's right. In combination it may be radically different.

DR. CHRISTIAN: Uh-huh. What we saw with that particular one.

MR. FREES [In Audience]: And that's the important point--

DR. CHRISTIAN: Sure.

MR. FREES [In Audience]: --for the registration of the product--

DR. CHRISTIAN: That's right.

MR. FREES [In Audience]: --for the sponsor. And the reason I bring this up is because, you know, the FDA is extracting opinions here. And the issue that they have to deal with is registration of the product. I have to deal with knowing what my adjuvant does, and whether or not it's toxic.

DR. CHRISTIAN: It's going back to the old thing: What is the question? And where are you in this stage of development?

MR. FREES [In Audience]: There are two different ones here.

DR. CHRISTIAN: Absolutely.

PARTICIPANT [In Audience]: Could I add something? I just wanted to say that it's not necessarily [inaudible]--

MR. : Use the microphone.

PARTICIPANT [In Audience]: It's not necessarily true that you'll never clinically study the adjuvant alone. Because there's instances in our own company where we have used adjuvant to compare reactogenicity. And so I think including an adjuvant-alone arm in some of your studies--I would be surprised that a sponsor would discover that their adjuvant is irritating in a repro study, though. You know, it seems that there should have been something before that.

DR. CHRISTIAN: Irritating the conceptus.

DR. VAN DER LAAN: I think what Dr. Frees indicated, that if he as a sponsor wants to know what the adjuvant does, it's also for me as a regulator important. And we have no different interests in that respect.

It's a little bit a "chicken-and-egg" problem: What's first? And you as a sponsor want to know, "What is the effect of the adjuvant? What dose should I use in combination with my antigen? And what are the toxic effects of the adjuvant alone, then in relation to the antigen?" And I think those are important questions that cannot be handled only in a combination between an adjuvant and an antigen. You should need, from my perspective, to have also data on the adjuvant alone.

MR. FREES [In Audience]: All good points. I'd only like to add one thing. If I have to physiochemically alter my adjuvant to study it alone, what have we learned?

MR.   : Well, yes, that might be a point. But I mean, think back to the list that the lady presented yesterday of the things that ought to be done to test the safety of an adjuvant. You know, there were some interesting "yes's" on there. One of the "yes's" was genotoxicity.

Well, I mean, do you want to do a genotoxicity battery every time you test a vaccine product of an adjuvant? You know, the easiest thing to do is to have the sponsor having done that, and make reference to it.

DR. GRUBER: I had a comment to make. I think we've discussed the issue of adjuvants--given by itself, in its own package, in a [inaudible] master file, combined with the vaccine antigen, and so forth--on yesterday. And I really see that we see all the points.

And regardless of how much trouble I'm going to get into here, I mean, I would like to stress that I think the points made by the audience here are well taken. You have to really ask yourself: What is the best information that we can get to clearly evaluate the safety of the final vaccine formulation? And the type of studies that we do should be driven by that question.

But if I may, I would like to get back to the discussions of reproductive toxicity study designs, per se, and animal models. I am struggling with how to really tease out and look at potential developmental toxicity that may be induced by potential intrinsic toxicities of the vaccine antigen or other components in the vaccine formulations; versus the immune response.

And what I think, what I have been hearing this morning and this afternoon, is that looking at the potential for--let's call it immunopathologic effects, for lack of a better term--doing the studies that we have been suggesting them to do in the guidance document, is probably not going to be feasible, given the differences in immune system maturation in the animal species that we have available to us for these types of studies, compared to the immune system and its maturation in humans.

So what do we have to do? Should we restrict developmental toxicity study designs to segment two studies, or extended segment two studies, in order to just answer effects on organogenesis or fetal development? Or should we extend the studies? Or should we do additional studies to evaluate post-weaning assessments, if this is what needs to be done to look at potential effects on the immune system of the offspring?

And right now, I'm really struggling with if we really should require that as a sort of one-packet approach, or if we should consider what was also mentioned by industry when we got the comments to the document. Should we consider a tiered approach, sort of looking at the developmental tox study as a signal-generating assay? And if we don't see any signals, we still can't say for sure, of course, that "My product is going to be safe when given to a pregnant woman."

Or should I go ahead and also look at immune response evaluations by doing additional studies? And that's something that I would like to have discussed, not only by the panel members, but I would also like to hear how the audience and industry feel about this approach.

DR. CHRISTIAN: Yes. If I can, let me just give you one thing that might be your first tier. And that would be, in the same species, compare the immune response in a non-pregnant and a pregnant animal, and see if there is a difference there.

And then, on a tiered approach, look at what we would usually look at in the parameters we can recognize.

Because even with first trimester insult in a rat or rodent model, we had the progenitor cells. Even if it isn't fully developed.

And you're going to get certainly the typical responses, with the exception of function, by C-section and certainly by postnatal day 21. You're going to see it as by ability effects and weight gain effects. They'll be noticeable.

If you want to add in some function, fine.

But that whole field is evolving from "behavior," which 20 years ago we finally got changed to "function." But now "function" isn't fully defined. And so that's an evolving area that will change with research.

What I would think is the first tier, if they had exposure during gestation and were allowed to go to weaning. That should certainly give you a pretty good initial tier one screen in one responsive species. And I would just suggest that as a good place to start. Then, if you see effects, you go to the next levels.

DR. GRUBER: Thank you.

MR. RUSSO [In Audience]: [Inaudible] Russo [ph], from Merck.

I think that the discussion was perhaps too focused on immune-mediated toxicity, to the extent that we struggle as it is to develop animal models that would be suitable for even assessing the efficacy of new vaccines that we are developing. And to really focus on immune toxicity--

[Tape Change.]

4A MR. RUSSO [In Audience]: --the way to do is to really assess whether there is this thing out there, and then trying to figure out how to do it. We have no discussion whatsoever here in a relevant model for cell-mediated immune responses. Most of the discussion was on antibody-mediated toxicity.

There was some presentation in terms of cell-mediated immune responses, but nobody discusses the host of genetic-controlled immune responses, even in animal models where parasites may skew the responses according to TH2 types, and so forth and so on.

DR. GRUBER: Yes, you are absolutely right. Just I wanted to answer this. I guess we are all a little bit uneasy to say that, okay, we're focusing, if you look at immune responses, at antibody response. But the reason why this is, is because we perhaps have the best assays validated and reproducible as is to look at antibody responses.

If you start discussing cell-mediated immune response, the question is: Where do you want to start, and where do you want to end? And do you want to throw in the cytokines profiles that you could potentially anticipate?

I guess I know that that is something that needs to be addressed in the guidance. I feel, though, that perhaps we should give some thought regarding how much and what to assess in terms of immune response. Should it go beyond antibody evaluations? Should it perhaps be driven by pilot or preliminary studies, to see what vaccine antigen do I have? What type of immune response do I expect for it to elicit?

Is it going to be more--Sometimes the adjuvants that I added will sort of shift the immune response from a TH1 to TH2. And so that you may want to say, "Okay, I'm going to look at certain cytokines, or certain cell-mediated responses."

But I'm not quite sure if we should build in as a first evaluation sort of a full assessment of the potential immune response repertoire. Because it could again lead us to data that may be very hard to interpret, at least in 2002.

I mean, I'd like to hear more comments. If you feel it's necessary to evaluate more than antibody responses, you know, we would like to hear this. But I think we're going to get--You had a question, a comment to make?

DR. HASTINGS: Well, Marion, can I follow up? This question is directed to Mercedes. For the therapeutic cytokines, have you got reproductive toxicology data for those?

DR. SERABIAN: Do we have it?

DR. HASTINGS: Yes.

DR. SERABIAN: More and more, yes, we do. We are generating data--mainly, seg two studies, teratology studies--with the cytokines. Again, it depends. The big thing there is antibody development, and basically clearance of the material. So it's basically not effective.

But what's your--Ken, was that your question?

DR. HASTINGS: Well, just to get at that. Because you know we were talking about other immune-induced molecules that might eventually be manifested as like teratogies.

DR. SERABIAN: Right. No, you do see some of them--Well, there are teratogenic effects with some of them, yes.

PARTICIPANT [In Audience]: Well, I'm struggling a little bit in the back here, so forgive me. This may be in part my naivete of this whole area.

But this is an extension of the gentleman over here. And he said when you talked about your tiered approach of looking at the toxicology or repro-tox assessments, can you, instead of looking at just gross or these larger changes that you're looking through, through the entire cycle of reproduction, connect it more to what is epidemiologically relevant?

I know the gentleman from EPA made some connections between effects that he saw in animal models and studies in Inuits, etcetera. I know that there are studies out there looking at immunotoxicological effects caused by the immune system in different ways when it's stimulated in different ways. And maybe one of the panelists could enlighten me on some assays that we could use, in this sort of area of immunotox, to really make a connection between the pathology or the function that we want to look for, and some sort of epidemiological feature that is a problem out in the population?

It seems to me like, if we're just looking at just any effect, as the gentleman said yesterday, you can create an assay just to create an effect. The issue is whether it's relevant or not.

DR. SERABIAN: I just want to, on top of that--I agree. I guess to me, when you mention this about tier, that sounds great, that's important; but again, what's the signal that you're looking for, and how appropriate is it, as to what testing you're doing?

And I guess, just kind of an editorial, when you say "immunotox" I kind of cringe. I think maybe you mean a module [inaudible]. I don't know. It's not always immunotox that you're looking for. It could be immunosuppressive. It could be immunostimulatory. And the word "tox" kind of--At least personally, I don't care for that. Okay.

Ken, do you have any suggestions as to the testing maybe?

DR. HASTINGS: Well, clinical immunotoxicology is a very poorly developed field. And there aren't that many things--I mean, you know, I guess the most important thing you would think about doing is just the prospective cohort kind of study, where you would look to see in the children, do they go on to develop susceptibilities to certain autoimmune diseases or things like that.

I mean, that's what happened, I believe, with cyclasporin. You find that there is a higher incidence of autoimmune disease in babies born to women who were taking cyclasporin. So I think mainly that's the kind of gross epidemiologic studies that you're kind of stuck with.

PARTICIPANT [In Audience]: I just wanted to make a comment. Marion, I think you framed the question really well. I've been a little confused by the discussion. There seems to be confusion between the immune system as the agent causing the toxicity, with the immune system as being an end point in the fetus for the toxicological effects of the vaccine. And I think you really need to separate those two issues in this discussion.

Yesterday, in the general tox studies, I think at least most of the consensus seemed to be that the general measures of toxicity were sufficient, and that special immunotoxicity tests as end points were not necessarily necessary. And I think that it is also true for our developmental toxicity tests.

Some of the talks this morning were very nice descriptions of the development of the immune system. And at the end of Dr. Barrow's test, he focused on the development of the immune system as to the timing of doses. But as toxicological end points, as the end points of these immune-mediated toxicities, we're not only worried about the development of the immune system, or development of the CNS, or any major organ system.

So I really think, as far as talking about end points, the emphasis should really be shifted away from the immunological system, and focused more generally.

DR. BARROW: I think the point was when we're dealing with vaccines, immunological endpoints, or in addition to all the other parameters we normally look at for other therapeutic areas,

PARTICIPANT [In Audience]: But I would ask why. Do you do that for drugs?

DR. BARROW: Yes, we do that for drugs. If we're testing a CNS-active compound, for instance, we pay particular attention to CNS development.

DR. SERABIAN: Okay, just real quick, I think that's going to be, hopefully, a focus of the next hour, or the end points. So we can continue with that.

PARTICIPANT [In Audience]: Sorry, one more question. I debated a lot whether to pitch this out here, but I'll just throw it just to see what happens.

The whole discussion about the potential issues related to vaccines and whether they cause a toxic effect to the animal model, to the patient, kind of leads to an interesting quandary. Some folks who work in vaccines feel that creating an immune response sometimes causes what some people would call a toxic effect; i.e., a swelling, redness, pain, sickness.

In some vaccine strategies, it may be a good idea to make a person a little sick initially, so that in the end they're protected from the infectious agents that actually may cause death or severe sickness or severe disease.

I wonder if by creating parameters like this we create vaccine strategies that won't impact a person's daily life, and won't make them sick, won't make them feel any pain;

but may not in the end be as effective a vaccine as we could possibly create.

DR. VAN DER LAAN: I think you're fully right, that developing or introducing a vaccine in an animal will lead to an immune response, and the immune response is a physiological one that leads to a lot of disturbances that we have discussed yesterday, too. We indicated that, also. The characterization of the immune response is more important than the definition or than defining or evaluating whether or not that response is leading to immune suppression or other things. The purpose of your evaluating the immune response is important.

With respect to the developmental aspects of giving a vaccine during pregnancy, it is important that introducing a vaccine may lead to an adverse effect. And your first effect is, of course, vaccination in the pregnant animal. But then it may also lead to an adverse effect on the fetus. And that's the problem that we are dealing with. And is the adverse effect on the fetus a direct abortion, or a malformation, or a functional malformation?

DR. CHRISTIAN: Yes. To carry that on, I think that the other thing that we have to have these types of studies for is ultimately in labeling. A woman is inadvertently vaccinated during pregnancy, and her question to her doctor is, "What should I do now?" And so we need to have some kind of indication.

If there are no adverse effects seen in these types of studies, at least the doctor can say, "We don't think it will be a problem." If we know that the response was such that the embryos died, then we can say, "Well, at such-and-such a multiple, we know that this occurred," also.

So remember that the adverse effect, even if it is a normal physiological response, can be a pharmacotoxic effect for the conceptus. And that's always the two sides of the concern.

PARTICIPANT [In Audience]: I guess what I wanted to just mention was--and it follows on nicely from that--I'm no immunologist, but it's my understanding that often in the first trimester, due to hormonal influences, women's ability to mount a cytotoxic T cell response is somewhat subdued. And we see that in terms of evidence of infection with toxoplasmic [inaudible], and other sorts of parasitic and viral agents like that.

So when it comes to developing a vaccine where we want to generate a robust CTL response, and a woman is inadvertently vaccinated in her first trimester, I think we would want to know, is there some model so that we could understand what would happen to the fetus? You know, is it going to cause an abortion? Because there must be some reason why we have a subdued CTL response in that first trimester.

And then I'll tell you, the other thing that really causes me concern is that we do these studies, and we do put something in the label to give the physicians some guidance. But we actually don't understand the influence of confounders, like women smoking through pregnancy and things like that. And then what does that mean, in terms of us getting sued because we've got something in our label?

And you know, we've done these lovely experiments in a controlled environment, and sought some understanding, and we're trying to provide some guidance. But it's also a very scary thing to sort of embrace, as well.

DR. GRUBER: Oh, yes. Yes. Yes. I guess nobody can argue with logic.

[Laughter.]

DR. GRUBER: But I think there's one thing that I wanted to actually throw out here. And that is, we do developmental toxicity studies for preventive vaccines perhaps in an attempt to be able to possibly identify potential developmental hazard, or using these studies as a signal-generating tool. I don't think that if we put the data into the label, that that is equal with saying, "Now we're going to make a prediction to human risk." Because everybody does understand that there is a difference between man and beast.

I think that, however, not having the data is really something that Francois discussed yesterday morning: It is sticking your head into the sand. And I think having some data is better than having no data at all. But I think that the difference between really predicting human risk, and using these as signal-generating tools, I think is an important difference that we need to keep in mind.

What I wanted to actually do before we break for coffee is--And I know this is not going to be a five-minute thing. But we've put a question up there. And perhaps in the discussions that Paul Barrow had we've already sort of answered these questions a little bit, and we discussed it a lot yesterday. But I think this is something that we should briefly turn our attention to.

And that is the question--Perhaps it's best framed again in: My animal model that I choose should be perhaps driven by the kinds of questions that I want to answer. And if it's really that what we're going to do here is a first tier evaluation, where I'm going to do a developmental toxicity study, and I carry this out to birth or weaning of the animal models, then perhaps I'm going to choose my animal model accordingly. And if I want to look at immunotoxic, immunomodulatory effects, I may have to look at another animal model, or do an additional study.

But how do we feel about the question about a relevant animal model? Can we define it by, as we naively stated in the guidance document, the ability of the species to mount an immune response? Or do we have to be a little bit more precise in our definition? And what other parameters do we need to consider?

[No Response.]

DR. GRUBER: Anybody?

DR. CHRISTIAN: Well, it certainly should get across the placenta, also. So you need both an immune response and crossing the placenta. Having only immune response, and it doesn't cross the placenta, it doesn't answer your question. Getting across the placenta without an immune response doesn't do it, either. So if you had none that did both, then certainly one of those would be better than none. But ideally, it should be both.

PARTICIPANT [In Audience]: I would only like to iterate a point that I made yesterday. It is that in this definition it's great, I think it's probably--The issue I have is, if you have an animal model, whatever model you select, that if you're using a vaccine modality that promotes an immune response in that species, you're probably okay.

If you're using a vaccine modality that doesn't promote an immune response in that species and is a very human-specific pathogen or vaccine modality, then you may be stuck. You know, you won't be able to address any kind of immuno-issues that are created by the vaccine. You may not be able to address some of these reproductive toxicology issues related to immune response.

You know, it's a real tough issue, because as we get more creative with our vaccine strategies, animal models may or may not become more relevant.

MS. MURSA [In Audience]: I'm Sandy Mursa [ph].

I guess I'm really confused. Because it seems to me that if what you're looking for is whether IgG crosses the placenta or not, then Dr. Barrow has outlined a nice way to figure that out. And you can determine that your animal model is probably a rabbit, and we don't have to carry on this discussion for very much longer.

But if that's not the thing, because it's hard for me to visualize--and I'm not an immunologist--but how simply IgG crossing and being available to a fetus is going to cause a malformation. That's pretty hard for me to understand. So I think that's probably not the point.

And then, you know, you say, "Well, if it cross the placenta," but I don't know what "it" is. You know, is "it" the antigen? Is "it" IgG? Is it--I'm not sure.

I don't think it's as simple as this. And if it is as simple as this, then I think you can just take and say a vaccine that mounts an antibody response against virus "X"; look for IgG; and then you never have to test another vaccine for virus "X" again. It doesn't matter what the construct is. It doesn't matter anything, if IgG is the only thing we care about.

DR. VAN DER LAAN: Let's try to go further in thinking. Yesterday I have indicated that in Europe we have thought about a relevant animal model as an animal model in which you can induce a change. But that's not always possible. And in this case, we have to use a case-by-case approach. Maybe if you have a polysaccharide vaccine, then the IgG response and the IgG transfer through the placenta might be very important. If you have a live attenuated vaccine, then the placental transfer of the virus is important. Or, as for small pox, it's thought to be that the interferon response might lead to an abortion very early in pregnancy. So it might be you should also in this respect use a case-by-case approach.

And then the criteria for what is a relevant model are much more derived from a comparison between the immunological response in humans to the infectious agent or the vaccine itself, compared to the animal model. I think that all those points have to be considered in this respect.

DR. LAMBERT: I would like to push the idea a little bit further. If we would like to develop a decavalent vaccine, and we should not be able to demonstrate immunogenicity in any species for the ten antigens, what should we do? Should that be a good excuse for not doing a study? Should we use a species where we have a maximum of immune responses?

[No Response.]

DR. VAN DER LAAN: We are all quiet. We have no answer. I think that's the most difficult situation, and it's very difficult to handle. But maybe there are people in the audience that would indicate, "Okay, animal studies are not necessary. You can directly go into man." I think that nobody has that opinion. But we have to struggle with that. I have no definite answer. It depends on the case.

DR. SERABIAN: Again, look behind you. I think that's one of the--Yes.

Any more comments with respect to the question I have up here now? No? Thoughts? Okay.

PARTICIPANT [In Audience]: Obviously, I'm working in an area where this is directly relevant, so--And the gentleman from Merck yesterday sort of helped answer this. You know, it's sort of a double-edged sword. You can switch to the animal system where--You know, like taking animal antigens or taking an animal-suited or a model-suited virus. Let's just take a virus, for instance, as a good example.

Let's say you're working with a human pathogen and there is an animal model, but it's not the human pathogen; it's an animal-adapted pathogen. You can use that animal-adapted pathogen, but you have to recognize that there's going to be big differences between the two, because it's an animal model and it's not going to be a perfect model.

But the challenge is, from a vaccine production point of view, that you have to develop these two things together, in the same manner, in the same way, to really adequately test the different relevant toxicological issues that may be related to these things.

And God forbid that the pathology or the pathogenic features of the animal model differ in any way from what happens in the human. Then you know, it's another whole issue to deal with.

So I don't know, it's sort of a very, very difficult problem. And I would love to hear folks who may have more experience in this give some sort of advice, because I think there's probably quite a few people in here who will be faced with similar problems related to this.

DR. VERDIER: I would just like to give one remark regarding the question behind me. I think it's really difficult to answer to this question without more detail about the specific vaccine. Because you have to consider the human data. Do we know something about the same infection in humans?

We have also to consider the nature of the vaccine. Is it a live viral vaccine? And in this case, the risk can be higher compared to a recombinant protein, for example. Do we have a strong adjuvant which can trigger a different production, or do we have no adjuvant at all?

So I think when we will write the non-clinical safety package in the IND or in the pre-IND, I think we have to take into account all of this information; and particularly, information regarding infection in humans. Do we have data which indicate that the pathogen can trigger abortion or can trigger cytokine release which can lead to abortion?

We were discussing with my neighbor about the different potential strategies according to the nature of the vaccine. And I think that if you deal with a live viral vaccine, it's very different compared to a recombinant protein. And we have to take that into account. We cannot answer "Yes" or "No" to a question. We have to take globally all of the information available.

DR. SERABIAN: I think that's an appropriate time to break. It's about 3:15. How long do you want to go? Till 3:45, then. Then we'll come back.

[Recess.]

DR. SERABIAN: Okay. Basically, I'd like to introduce the two remaining people on the panel here that have not been more formally introduced.

Dr. Verdier, I don't think I need to read his introduction, since you know him quite well from yesterday and today. And he will give a very small presentation--two or three slides, I think--to just start us off.

And Dr. van der Laan, he is a pharmacologist and toxicologist. Since 1990 he is head of the preclinical assessment group of the Medicines Evaluation Board of The Netherlands. It's located at the National Institute for Public Health and the Environment. In this function, he is responsible for giving advice on preclinical safety aspects for The Netherlands College.

On behalf of the Medicines Evaluation Board in The Netherlands, he is a member of the Safety Working Party, the SWP, of the CPMP. In the SWP, he was responsible as a rapporteur for the note for guidance on preclinical, pharmacological, and toxicological testing of vaccines, as well as on the revision of the note for guidance on repeated-dose toxicity, which included immunotoxicity aspects.

IMMUNOLOGICAL ENDPOINTS

PRESENTER: FRANCOIS VERDIER, PHARM.D., PH.D.

PRODUCT SAFETY ASSESSMENT, AVENTIS PASTEUR

DR. VERDIER: Thank you, Mercedes. I will just briefly introduce the subject about what are immunological end points. And I think we have to ask the following questions.

With the immunological end points, we want to confirm the relevance of the animal model. And we were speaking about surrogate markers, antibodies in the mother or in the fetus. An antibody measurement can be used as surrogate marker to confirm that the animal model is partially relevant.

We can use also immunological end points to evaluate potential adverse effects. And we will see when and how.

These immunological end points can concern the mother, the fetuses, or the pups, on any other model.

To illustrate these immunological end points, I would like just to show you this graph which represents the cytokine balance during the pregnancy. And it's true that if you interfere with the cytokine equilibrium, you may induce pregnancy loss. So you can imagine that if you give a live virus and if you trigger high production of interferon, you can perhaps impair the pregnancy.

So about immunological end points, I don't know if we should measure the cytokines, but at least we can imagine that if we give a strong stimulus, if we give a live virus which will really trigger a strong cytokine change, you may have changes in the pregnancy.

Regarding surrogate markers, I think when we are measuring IgG we are not measuring IgG for the potential toxic effect. We are measuring IgG to show that we are triggering something to show that we have selected an animal model which answers to the vaccine.

And I have just reproduced here what we are doing. And Paul presented this kind of treatment design in his presentation. In fact, we are immunizing the animal before the mating, during the gestation period. And also, for some subgroups we are doing postnatal vaccine administration. And we measure the IgG just to show that the species is answering to the vaccine.

So for me--and I hope that you will challenge this idea--in a tier one, we do immunological end points to justify the species selection and the protocol design. Only for that. And we will do the study without immunotoxicology tests. We mainly focus on classical teratology end points. We don't do cytokine measurement. We don't do functional assay by immunizing the animal with another antigen.

I should admit that we don't re-immunize the pups with the vaccine, because we know that maternal immunization will suppress during a certain period the answer of the pups to the same antigen. So we don't re-immunize the pups. It is mentioned in the guideline that we may have to re-immunize the pups. Until now, on the four studies we performed, we never did that. So perhaps it's something which should be modified. I don't totally agree with that, but it can be discussed.

Regarding the tier two, I would limit immunological end points to mechanistic investigations. And I have in mind only one example, which is meninges-B polysaccharide vaccine. In this case, in addition to a classical reproductive toxicity study, you may want to do in vitro antibody binding to show that the antibody made by the vaccine can bind to fetal tissue.

In another study, you may want to show that your adjuvant, or your live virus, perhaps can trigger a cytokine change. But I would keep these investigations in very specific cases: only if we have some good evidence that the vaccine or the adjuvant can trigger some changes, and if we want to further explain these changes. But I would not do this very specific investigation in a first tier.

I will stop here, and I will let my colleagues from the panel or from the room comment on this proposal.

[Pause.]

DR. VAN DER LAAN: Shall I first give my statement, and then we have the general discussion?

DR. VERDIER: Yes. Go ahead.

DEVELOPMENTAL ENDPOINTS

PRESENTER: JAN-WILLEM VAN DER LAAN, PH.D.

DIRECTOR, PRECLINICAL ASSESSMENT GROUP,

MEDICINES EVALUATION BOARD [RIVM], THE NETHERLANDS

DR. VAN DER LAAN: I have not prepared a presentation as the other chairpersons for the sessions. I have only one point that I specifically want to bring in the audience, and a point that we have discussed repeatedly in our European clubs--the Safety Working Party, and the Small Pox Working Party--early this year.

And I think it's important that reproductive toxicity testing is not a purpose in itself. And that's important. Vaccines are derived, by definition, from infectious agents that cause human diseases. And to get insight in the risk of vaccination during pregnancy, we can learn a lot from the clinical experience with the pathogen exposure.

So for the live viral vaccines, as influenza, rubella, the mumps, the measles, and variola, the human pox--there might be others--we can learn a lot from the epidemiology from the illness itself.

And then, we have to think about, if the complete market will market the specific vaccine, what will be the decision for the treating physician? And as the U.K. is part of Europe: To treat or not to treat? That's the question. And that depends on the situation. Sometimes, passive immunization during pregnancy is more important than giving a vaccine. And we should that clinical background keep in our minds when we are discussing reproduction toxicity testing.

That's just another aspect. And with respect to the other developmental end points, just because of these types of examples we know the developmental effects of rubella and human pox. I think those are not based on the--And Dr. Holladay is not present here behind the table, but he explained that that type of effects might also be immunological effects. But those types of end points are, of course, still important in reproductive toxicity testing.

Anybody from the audience has any comments on these statements from Dr. Verdier or from me? Or anybody from the panel? Yes.

MS. SHEETS [In Audience]: Hi. I'm Rebecca Sheets. And I just want to be clear to everyone in the audience: I'm not longer at FDA. so I'm not speaking for FDA.

It is my impression that animal models--I mean, we've had a lot of discussion about what's a relevant animal model, and how difficult that's going to be. It's my impression that animal models are inherently imperfect, and they may or may not be predictive of the human situation. So to expect the animal models to predict subtle effects, like the immunological effects, it's going to be asking too much of the animal models.

I think it's warranted to do these kinds of studies and to be looking for gross effects. And if you see such gross effects, then doing further studies in a second species or that sort of thing may be warranted. But I think the only way to get at these subtle kinds of effects is really going to be studying humans and epidemiology. And, yes, there's a lot of problems with doing epidemiological studies, as well.

But I think that it's asking too much of these imperfect animal models to be looking at very subtle, downstream effects that may or may not be seen, may or may not be able to be measured, and in the end may or may not be relevant. I think looking for the gross effects is really all we can ask of these animal models. So that's just my scientific opinion.

DR. VAN DER LAAN: Anyone from the panel? Marion?

DR. GRUBER: I'll hold my comments.

MR. PARKMAN [In Audience]: Hi. I'm Paul Parkman. I listened all day yesterday and today. And it seems to me that from what I've heard, the evidence that past vaccines are toxic, either reproductively or developmentally, in a way that preclinical laboratory studies can help, is extremely rare. Rubella, of course, is one of them. It seems to me likely that the need for these tests is driven by the need to have something we can say in the packet circular about these matters. And given this, I think the most useful approaches might be two-fold. One is, in the unusual circumstance where there is some reason, from epidemiology or clinical medicine, to suggest concern--and rubella might be a classic example of that--then the sponsor should be required to develop studies that are tailored to answer the specific questions that are raised. And so some sort of screening test wouldn't be particularly applicable here.

For everything else, it seems to me that a toxicology test should be sufficient in one species, using the "best animal model"; recognizing that often the best model is probably not well defined.

But for these studies I would think probably reproductive toxicology would not be required, unless there was some new and really convincing evidence of a certain need for them. That would be sort of my take on it. Thank you.

DR. VAN DER LAAN: Anybody, comment?

DR. GRUBER: Yes. I have a question for Dr. Parkman. How would you define evidence for the need of developmental toxicity studies in the absence of clinical and preclinical data?

DR. PARKMAN [In Audience]: Well, what I was referring to as evidence was evidence from epidemiological studies of the disease or a clinical study of the disease that suggested that the organism or organisms closely related to it had some reproductive effects that it was important to define.

DR. GRUBER: Well, thank you. That's, of course, one point of view: To have reproductive toxicity studies only for those types of products for which the "Y" type disease would suggest an untoward effect on fetal development. However, as we have been pointing out, we're really faced with a really novel area of vaccines, product classes, combinations of products, the introduction of novel adjuvants; that I think that we may be going down a dangerous path to really dismiss all these issues and just look at "Y" type disease. But that is my personal opinion, and I guess that is something that we can discuss a little further.

PARTICIPANT [In Audience]: I think that one of the problems that the audience has been grappling with is this almost necessity to have one type of study fits all cases. In reality, we could look back and say with our history of vaccination we really have no history of repro-toxicological problems.

However, we're all very excited, because we're facing a whole new era and set of opportunities in developing vaccines. And we're trying many new approaches. Maybe in tailoring these guidelines and so on we have to take that into consideration, that in a situation where we're using live viruses, attenuated live viruses, one has to look about transfer.

If the goal is to use cytokines as adjuvants, then measurements of cytokines would be relevant. And maybe we really are going to have to consider this based on the different categories of vaccines that are going to be developed.

DR. VAN DER LAAN: Anyone to comment on this?

[No Response.]

DR. VAN DER LAAN: I think you gave a differentiation, but you have given maybe voice to the audience that you agree that we are going this way as regulatory authorities in setting up these guidelines, providing this guidance to the industry.

Are there other opinions in the audience not willing not follow this guidance?

MR. HOPKINSON [In Audience]: Hi. I'm Bob Hopkinson [ph], from DynPort Vaccine Company. I'm also no longer with the FDA.

I just wanted to comment. I don't have a strong opinion in this area, but just in the world of drugs where I was before, you talk about what are the implications in terms of the label with these studies.

And one area that comes to mind is the quinoline antimicrobials. Early on, multiple species tested, finding cartilage toxicity. Getting into the label--Products never being used in pediatric populations, or very infrequently being used, and the use essentially off-label for years. And it's only recently with resistant pneumococcal and other types of infections where FDA is being asked to consider looking at pediatric studies and trying to get some additional information.

Epidemiology studies really can't be done if you've got something on the label related to an animal toxicity which may or may not be relevant. And so, just another thing to think about in terms of our thinking process. If we search for a species that may cause an effect and we find it, okay, then you have to decide, well, does that mean anything? And it may preclude actually getting any epidemiologic data, because no one is willing to use the product in a pediatric or gestational period.

DR. VAN DER LAAN: I think that that's indeed an important statement; that you can also abstain from giving a vaccination during pregnancy. But the problem is, as indicated by--

[Tape Change.]

4B DR. VAN DER LAAN: --this risk assessment, where you can not always avoid it.

DR. CHRISTIAN: I wanted to say that I agree with your tiered approach. And I think that to look for specific end points that are functional without a reason, in an initial run-through, with no other effects, would be pushing the model perhaps beyond what we can do at the first tier for screening.

But I believe that that first tier is important to do, because we don't have good data as a rule on the disease models themselves. And we're coming up with so many new things that it isn't just the immune response, which was what we were first looking at, if you could even define what we were considering an immune response; but rather, the multiplicity of the different types of agents that we're using.

And I don't think, or I hope nothing was interpreted as "There's only one way to do this." It's certainly a case-by-case basis, where the sponsor is responsible to figure out what they know about the compound and what's the most appropriate way to test it. And I think that's just axiomatic, and should not be forgotten.

Now, if they have a reason they think it is going to be immunotoxic or immunosuppressant, then you test for those things, just as you would if you thought it was CNS-selective and doing something there, or toxic to the liver or the kidney or something else. You would put in any points that you wanted to look at to identify effects in the adults.

But I believe that ethically, before we go into pregnant women or have inadvertent exposure of pregnant women, we have to do the best test using the current tools that we have--admitting that they are inadequate; but they are still better than nothing.

DR. GRUBER: I couldn't agree more with your statement, Millie. And I really also wanted to say that I am supporting Francois' suggestion for the tiered approach that is no longer up on the screen.

The question, however, that I feel we somehow have to answer, coming back to what I said this morning--We wanted to hear comments; we wanted to address concerns raised by industry in response to us publishing this guidance document. At the end of this day I'd say: Are we back to the ICH as far as a guidance document? Do we need an additional document at this point?

So I see people shaking their heads, nodding. Jan, you wanted to say something?

DR. VAN DER LAAN: I think that vaccines are in their concept so different from conventional products that it might be helpful to the industry to give guidance in addition to the reproductive toxicity testing document from the ICH.

I'm wondering why only at this point the FDA has made a guideline. But I would say, have a guidance document. And I have learned that you are preparing that for the development of vaccines.

I have a question also on the tier two to Ken Hastings. As we know, in the immunotoxicity discussions for the conventional product, we have given a first look at the developmental immunology of immunotoxicity testing; and in that way, a function test at day 21--day 20, 21, or the period of weaning.

What is your feeling? Should that be a standard approach for vaccine?

DR. HASTINGS: Actually, I was thinking about the one slide that Ralph showed, Bob Chapin's very complicated but nice repro-tox testing scheme. And it did have the immunotox end points.

As you know, in the immunotoxicology guidance we say that if you know that a compound is immunosuppressive and you know it's likely to be used in women who might become pregnant while taking the drug, that there should be an evaluation and a repro-tox study, and basically we said a histologic examination of immune-related tissues. And we kind of left it at that.

And the reason for that was that we felt like when we were writing the guidance that there wasn't enough information to make a recommendation about a functional assay. Now, Ralph and some other folks have actually worked very hard to develop these functional assays to be incorporated into repro-tox studies. And I would like to see a lot more work, or some more work, done to that, so that maybe we could make that recommendation.

And I think that the work that Ralph and Greg Ladix [ph] and some other folks have done purports, you know--I won't use the term "validated," because that's a heavily weighted term. But where we could feel more comfortable about that, then, yes, then at that point I would like to see that incorporated. And we probably would change the guidance at that point.

Did I answer your question, Jan-Willem? Yes.

MR. RUSSO [In Audience]: I'm [inaudible] Russo, of Merck. I'm not sure that I understand the logic behind that. Because you say if you have any reason to believe that the drug that you are developing is immunosuppressive, then you do this recommendation. I guess it's because you want to assess whether or not this temporary immune suppression will affect the fetus by exposure to viruses or microbiological agents. Is this really relevant to vaccines?

DR. VAN DER LAAN: May I give that question to Francois? Do you expect that you ever will apply the second tier testing?

DR. VERDIER: I would not include a functional test like suggested by Ken. Sorry, Ken.

DR. HASTINGS: That's all right.

[Laughter.]

DR. VERDIER: I think at this stage we want to have some gross evaluation of the vaccination on the pregnancy. It seems that we are in a totally different situation compared to chemicals which can trigger an immunosuppression. So I would be cautious about adding functional tests at this stage.

And that's why also, I think I was clear in my presentation, I would not re-immunize the pups with the same vaccine. Because I think this can be misleading. We will observe a suppression of the B cell response, and some people may think that it's an immunosuppression. In fact, it's not an immunosuppression; it's a normal effect of vaccination of pregnant animals or pregnant women.

So I would avoid to add either an immunization with the same antigen, or I would avoid also to add function assays. But that's my very personal opinion. I think it's the opinion, also, of my colleagues from Merck. Perhaps in 20 years we will have a different opinion, but today that's it.

DR. VAN DER LAAN: Thanks.

Marion?

DR. GRUBER: Yes. I just wanted to make one comment, and I think that is an FDA comment. If you read the draft guidance, I think the issue of re-immunizing pups to further look at potential for immune suppression is something that even the guidance document did not really support.

We really said that these types of issues may need to be addressed clinically. And as a matter of fact, there are instances for maternal immunization studies where the potential for an immune suppression in infants is addressed clinically because we didn't feel that the animal models would really give you the answer to that question.

DR. CHRISTIAN: Just a comment on your question of: Do you need guidance other than ICH? I think the real problem is that the ICH guidance covers everything, but here we're not looking at a standard type of response. Because we're really testing the effect of an immune response on the pregnancy, rather than in combination with an adjuvant or whatever other things that are in this particular vaccine. And it would be helpful, because these groups generally could use the guidance. And it would save you some telephone calls, perhaps. And they would have it in better order when they come to see you, because they'd have guidance; rather than saying, "Oh, I'm going to do it every day because that's what's appropriate for a developmental tox study," or, "I don't know that I should look to see whether it crosses the placenta," and so forth.

So I think the guidance document would be helpful, particularly because there are so many new companies that are coming along; where the large companies, they've got their programs in order, but the small companies need some guidance.

PARTICIPANT [In Audience]: [Inaudible] from NTI Research. I'm a veterinary pathologist.

And going back on the same topic of going back to the ICH guidelines, I would like to hear some rationale for actually even measuring antibodies on the mother and the pups or in the milk. As a pathologist, if you're concerned with the adverse effects of antibodies or toxicity, and not efficacy, but if you're concerned with toxicity of antibodies you would look for effects in the fetus by histopathology or post-weaning. So you do multiple time points. Because just measuring antibodies won't tell you anything.

And I'm seeing myself writing a report of antibody levels and going, "Okay, there's antibodies in the serum of the dam, there's antibodies in the serum of the fetus--" Or, "There's no antibodies in the serum of the fetus." What do you do with that data?

You know, I understand Dr. Verdier's point of, okay, you're proving that you're inducing antibodies and the antibodies are actually passing to the fetus. But it's almost like a given. I know you don't assume anything, but you get a rabbit, that is expected that 100 percent of antibodies in the serum of them will pass to the fetus. I still don't see what you do with that data.

Okay, let's say you look at--And then there's antibodies positioned in the tissue of the fetus. If there's no damage, what do you do with that data? So I think I keep going back, and I don't see a reason, unless anybody can give me a better rationale for that.

DR. BARROW: Just to make sure I've understood, if you don't advocate looking at antibody titres, what other measure of exposure would you use?

PARTICIPANT [In Audience]: Well, you have all the data. You have your efficacy data showing that you can induce antibodies in adult animals, right? And so I'm basically just assuming that if you have a 100-percent transfer of antibodies, it's a passive transfer; it's not an active process.

DR. BARROW: It is an active transfer. It will depend on your vaccine in question.

PARTICIPANT [In Audience]: Okay. So I guess you could use that to prove, but I still don't see in the end what you do with the data. Like, okay, we proved that it did transfer. And what if it doesn't transfer? Then you have to re-immunize to make sure that you have antibodies in the fetus?

DR. BARROW: If we suspect there will be exposure in the human, yes.

PARTICIPANT [In Audience]: Okay. Thanks.

PARTICIPANT [In Audience]: Are we worried about the exposure to the antibody, or the intended immunologic consequence of the immunization? Or are we trying to assess the toxicity associated with activation of the immune system and what effect it will have on the conceptus, on the dam carrying the fetus to term, those kinds of questions? Those are two different things. We're talking about inadvertent immunization of a pregnant woman at some point in pregnancy. I don't know whether you should be doing the immunization during gestation. The issue is--

DR. BARROW: No, that is a--I'm sorry, can I just interrupt?

PARTICIPANT [In Audience]: Yes.

DR. BARROW: That's a consequence of the different gestation lengths between human and animal. We have to vaccinate--

PARTICIPANT [In Audience]: But the question is the effect of, let's say, the cytokine milieu after immunization. That should be done during gestation. And the primary cytokine milieu from a primary challenge may be different than a secondary challenge.

So I understand. Measuring IgG, and that tells you that that species can make an antibody response. And if you're worried about whether that antibody is going to cross and cross-react with some fetal tissue, that's a question, and certainly that makes sense.

But if you're worried about inadvertent administration to a pregnant woman, that's a different question. Then we can go down the path of saying, oh, it could be different on any given day of gestation. And then none of these models really address that question. So I'm still back at: What is the question?

DR. CHRISTIAN: I think Paul will probably back this up. The idea of getting to the maximum insult, the maximum exposure, and to have that over the extended period of gestation, at least from implantation to, let's say, the end of the fetal period, that's to address inadvertent exposure, by having that maximum response over all of those different days.

The only other way to do it is to do it on each of those days, which is the approach sometimes taken when you have two or three days, and then you do it another time during gestation, and two or three days. The other question, though, is if it's intended exposure. And that's a different case.

PARTICIPANT [In Audience]: Well, intended exposure if it's during--You know, again, the primary should be given during? You may do another arm where--

DR. CHRISTIAN: Yes.

PARTICIPANT [In Audience]: I think the GSK person mentioned doing where they immunize prior to, and then have another group where they do it on day six only. That seemed like a reasonable model approach to me, also.

But inadvertent administration--Again, are we worried about the intended consequence, the high-antibody titre and its effects on the fetus? Are we worried about the bystander effect of the adjuvant and the hyper-immune response that we're trying to induce to get that antibody response?

DR. VERDIER: I think we worry about both. My first feeling is that the first risk is an interaction with an immunostimulation which would trigger something abnormal in the pregnancy status. That's my first fear.

But in some cases, perhaps very rare cases, antibodies can perhaps have a harmful effect, as is the case--question mark--with perhaps meninges-B polysaccharide vaccine, even if we have never been able to show any relation with these antibodies.

DR. VAN DER LAAN: May I add a question in this respect? Do we need really the measurement of the antibody in the fetus? If we know, based on the data that Paul has shown and data known from literature, that certain types of IgG will cross the placenta, the industry has to prove that every time again, if there is no further consequence to be expected?

DR. BARROW: I think we need that data to justify our choice of species. As you saw with the data I presented, with different vaccines we did find different levels of maternal antibody transfer. So we used that data to justify our choice of species for the main study.

PARTICIPANT [In Audience]: I have actually a very provocative question. When I think about the inadvertent administration and the reality that the animal studies-- Basically, animals lie, and you can't really rely on a lot of the data that you get from animals.

So the provocative question is: Is the information that you're going to get from the animals more relevant or more useful than the information you would get from the pregnancy registries? And maybe the pregnancy registries should be something that is pushed more. Very provocative question.

DR. VAN DER LAAN: Very provocative. Who wants to give a first response?

DR. GRUBER: I would like to give a response. I don't think that this question is provocative at all. I really think that we need both assessments. I really don't think that we can do away with developmental toxicity studies and wait until we have exposed pregnant women to get pregnancy registry data. I think we have to attempt to address the potential for any adverse effects of the vaccine induced in a potential pregnancy situation with all methods that are available to us. And in my opinion, that includes preclinical studies.

DR. VAN DER LAAN: I will add to that that, indeed, in this way, as a company, you are requesting for every physician treating pregnant women to do an NS1 study, without any control. So that's the real background.

We have to be aware of the fact that we are not developing guidelines for the old products that are reasonably well characterized thus far. But we are developing or writing guidelines for products which in many cases are recombinant vaccines or genetically changed, and that type of stuff.

So you first have to characterize that type of risk. And it's not very ethical to do that directly into humans.

MR. THOMAS [In Audience]: Larry Thomas, Avant Immunotherapeutics.

A lot of the discussion has centered on the assumption of a per enteral vaccine. I was just curious about the feeling of the panel on if there would be any expectation of different end points or design for a mucosal vaccine? Assuming of course that there is a case-by-case assessment.

DR. VAN DER LAAN: Who will take this question?

DR. GRUBER: I don't have an answer. I can just tell you that we're going to be discussing this question, if we should really be requiring developmental toxicity studies for vaccines that are mucosally administered. We'll be discussing that, but we haven't really been arriving at a conclusion.

I guess the point, again, is made, you may have a mucosal exposure, but you may also then get systemic exposure. And again, you will have a systemic immune response induced. And so I think you can make a case for requesting a developmental toxicity study.

But I think that goes a little bit into the area that I don't think that we can really discuss here. But I think there is one question that the agency also has to discuss. And it is really taking another look to say, "Do we really need it for every product? Or could there be cases where there are exceptions to the rules?" And I think we need to discuss it. But at this point, I don't think there is any regulatory stance that I could give you.

MS. HOLMAN [In Audience]: Lisa Holman [ph], from GlaxoSmithKline.

Yesterday I asked a question about multiplasmid vaccines. And I was told that for toxicity testing we would need to consider those individually. Well, for repro-tox, when we look at recombinant vaccines and live viral vaccines, what we're looking at is the mixture of epitopes, maybe T cell epitopes. And we mount a polyclonal humoral response.

If our developmental studies focus more on the immune response for multi-component DNA vaccines where we are going to mount a cell-mediated immune response to a variety of different T cell epitopes and the polyclonal response, isn't it more relevant to look at it as a whole product when we are looking at antigenic competition; and take Francois' tiered approach, that if we do see something with the combination product, that we then go back and look at it mechanistically in a single plasmid situation? Could the panel comment on that?

DR. VAN DER LAAN: Who is taking this question? Francois?

DR. VERDIER: For me, it's quite obvious that we are testing the final vaccine with the adjuvant with a different component of the vaccine. And then, if we find something, we can go further. That's all I can say.

DR. GRUBER: Yes. I would have to think about this a little further. And I don't know if--I probably don't have a good answer here right now. But in a way, I mean, why are the issues so different at that point? Maybe I just don't understand your question right. But, yes, I don't know.

DR. VAN DER LAAN: Can you give why your problem is different from what we have handled thus far?

MS. HOLMAN [In Audience]: Well, I tend to think that there is a case for actually testing the whole vaccine in a repro study. But I guess I'm answering my own question in that, if I were to ask the question of were we going to have to do repro-tox on separate plasmids.

The question yesterday was we're going to have to do repeat-dose toxicity on separate plasmids, even though they're going to only be administered, ever, in a single product. So with the answer to that question yesterday, I'm guessing that the response will be that they will want individual plasmid repro-tox data. And I don't think it's relevant to generate that. So I'm asking you to consider whether, as a repro-tox panel, you think it's appropriate to test them separately, or as a combination product.

DR. VAN DER LAAN: Is that a different answer? I would suggest that we should know more from this product, to give a more precise answer. Apparently, you have some of your concept in your mind that's not easy to explain in this way.

DR. GRUBER: That's perhaps true. And please, do not take this as a regulatory position, but if you are required--and we heard this yesterday--to do separate preclinical studies to evaluate the safety of the plasmid [inaudible] and then the plasmid containing the antigen or genes for the antigen of interest, you have already then that battery of preclinical data. And so then it would be conceivable to me that you can go into the reproductive toxicity study with your full product, because you have the other preclinical data. Okay? But you know, this is a very novel question. And we will take this into consideration.

MS. HOLMAN [In Audience]: Thank you.

DR. VAN DER LAAN: There were two questions there. Yes.

PARTICIPANT [In Audience]: Yes. Regarding the registry, I think that can be done as part of the clinical development. So as we do at Merck during the development, we collect data in pregnancy, and that can be used at the end before licensure to provide and list the initial database on that. The second comment is regarding your question of whether or not we should measure antibodies in the fetus. And I'm not convinced of the relevance of any animal model that we're going to use. And so I don't really know how we're going to extrapolate the data you're going to get in any animal model to what's going to happen to people. And so, I'm not sure this is going to help you at all.

DR. GRUBER: Can I give this a shot?

DR. VAN DER LAAN: Yes.

DR. GRUBER: I think when we wrote the guidance and we said you should evaluate, or you should look for antibodies in the fetus, I think where this was coming from is from vaccines indicated for maternal immunization where you really want an antibody transfer to the baby to protect it from neonatal disease.

And I think the ability to also demonstrate antibody transfer then in an animal model from the dam to the fetus was really like a proof of concept issue, to say that you can demonstrate that you are able to show this; you know, keeping in mind, of course, the limitation of an animal model.

But Carlo, don't you face the same problem if you develop a vaccine candidate, some preventive vaccine that you give to a non-pregnant population, and you do your proof of concept study in an animal model to see that your candidate is immunogenic and has the desired effect? And I think that's sort of why we wrote it that way.

PARTICIPANT [In Audience]: Right. I do understand the question if it is an efficacy question. I don't understand the question if it is a safety question. So I understand why you put it, because you want to make sure the intent is to have an antibody in the serum of the fetus. So it makes perfect sense in that case to go and test it, because that's in the intent.

But if you're just fishing for toxicity, it doesn't make any sense to me to go and look in the fetus, because I'm not sure the data are relevant. But I understand your point.

DR. VAN DER LAAN: Yes, I can agree. I have the same feeling in asking for the toxicological elements of this. First, is this so, what Dr. Barrow said, the exposure? And exposure can be different from different vaccines. And the second point is then what Marion now indicated, that the exposure might have also effects that you want, intended effects in the neonate.

Other question?

MS. SAEGER [In Audience]: Polly Saeger, from NIAID/NIH. Maybe I've missed something here today. And it's entirely possible I did. But I'm thinking about, you know, we're in the government; we're helping various sponsors develop vaccines. And I'm right now responsible for setting up some of the resources to help with development of biodefense vaccines.

So I'm thinking, we're setting up these assays that would be required before we would go into phase III trials and whatever. In my experience, before when we were setting up assays, we've gone through a phase of trying to validate our assay, or at least make sure it's standardized. And part of that includes looking at negative controls and positive controls.

And what I haven't heard here, I don't think, is what I could use with working with my investigators and contractors in setting up these assays as a positive control that would be appropriate for testing vaccines. I mean, did I miss something, or is there a vaccine or a vaccination schedule that can be used as a positive control in this kind of repro-tox assay?

DR. VAN DER LAAN: Your question is a double question. Referring to the use of a positive control and for the positive control standard, for that positive control I think every vaccine has its own schedule. But for that vaccine you have a standard--

MS. SAEGER [In Audience]: No, no. No, I'm not talking about--When you're setting up an assay, okay? So if someone has not been doing this before, necessarily, or I'm hiring a contractor to do repro-tox testing on an anthrax vaccine, okay? If it were a drug, I would ask them to show me data that they have been able to show a positive effect from some standard, known drug that causes the developmental toxic effect in this assay, so that I know their assay works. Because you have to be able to show an effect in a study.

Ken, do you know what I'm talking about? So for the vaccine studies, what would you recommend as a positive control to be used in the assay?

DR. HASTINGS: Well, in a standard repro-tox study for drugs, you don't use a positive control.

MS. SAEGER [In Audience]: You don't. But before I would hire someone to do that, I would want them to show me data that in their hands they can get a positive result.

Correct?

DR. HASTINGS: Right.

MS. SAEGER [In Audience]: So if you received data from someone you'd never looked at data from before, you would want to see that. So I mean, if you want to set this up and do it, what can we use? Other than rubella, are there any others?

DR. VAN DER LAAN: As far as I know, your question is a validation of the model.

MS. SAEGER [In Audience]: Exactly.

DR. VAN DER LAAN: And I think there are more people in the audience that are asking for that. I think that in all the discussions that we've had on the relevance of the animal models, that that's a very difficult issue. If you have to sponsor some researchers, I think then you have to keep in mind that such a particular safety study should be done under GLP. And so you should go to a company that is able to do a GLP and has control data and so on, and is doing the right job. That's my interpretation.

MS. SAEGER [In Audience]: I understand all of that.

DR. VAN DER LAAN: Yes.

MS. SAEGER [In Audience]: The question is, other than rubella, is there any evidence that any other vaccine tested in this kind of system has caused a positive effect in the kinds of developmental tox studies we have seen here?

I believe Dr. Christian showed one that you said was related to the adjuvant.

DR. CHRISTIAN: Yes.

MS. SAEGER [In Audience]: And what I'm asking is, of all the other studies that people know about that have been done, can anyone give me--I don't want the details, but tell me, have there been ones that are positive, weakly positive, strongly positive?

DR. CHRISTIAN: Not that we've done. And we've probably done the most, so I guess you can't even use us. But I would say that what you want to look for, if we're going to restrict it to the usual developmental tox end points, you want to know that the lab historically has experience conducting that; that they've worked with the species and can observe those end points in that species which is responsive to your vaccine; and that they may have other compounds that show similar things.

I was just thinking, for example, if we're looking for immune response, you might even look at something like, do you have evidence of uncoupling agents, for example, which cause fevers in animals. I could show you that and say, "Well, this is one potential thing that could happen as the result of the vaccine, and here is an effect of having a fever."

But it would be very difficult, since we don't have a vaccine, a therapeutic vaccine, that in my experience--and I don't know, maybe Paul has one, or one of the companies has one--that has had an adverse effect. It would be awfully difficult to do that as a positive control. To the best of my knowledge, there isn't one.

And the same applies to drugs, though. Because having a positive control drug merely identifies that you can identify some end points that change. It doesn't necessarily mean anything at all relevant to the new drug entity.

MS. SAEGER [In Audience]: Precisely.

DR. VERDIER: Just to answer indirectly to your question, I think we have some data regarding administration of cytokines in animals.

So Paul, I don't know if you want to comment. But it's not directly a vaccine, but you can imagine that your live virus will trigger a cytokine release. And we know that cytokines in mice, and also in humans, can trigger abortion. So it's indirect proof.

MS. SAEGER [In Audience]: Okay. But what I'd like to suggest, then, is that maybe FDA in their guidance set this up to take a look again at this after some period of time, that if we're doing--everybody, all the sponsors, are doing these repro-tox studies--that after a period of three to five years, or "X"-number of vaccines in specific categories have been looked at, that the FDA reevaluate whether or not to continue to require the studies.

Because I could see that for some categories of vaccines it could be a real issue. For other categories of vaccines you may find that there is no evidence after "X"-number of vaccines through that you've ever seen anything; in which case I would think you might want to reconsider it as an absolute requirement, and do it only as a case-by-case basis.

DR. GRUBER: Yes. But now you raise two entirely different points. But I think we all agree with your last statement made, that with experience comes wisdom, and at that point we can reevaluate our approaches. And I think FDA has been doing this all along. But your point is well taken.

DR. BARROW: I'd just like to add one point. Why are you considering vaccines to be different to any other therapeutic class? Would you, for instance, say when you want to place a study to test an antibiotic, would you say, "I want to see positive studies with another antibiotic" before going ahead?

MS. SAEGER [In Audience]: Personally, if I'm going to spend money on a study, I want to know that the person, the group, that's doing the study has positive results. I think we know for many of the drug classes, if not all of the drug classes, or a whole bunch of them. I mean, the reason you do repro-tox is because things have come up positive.

Now, whether or not that totally correlates with what you see in humans is a different story. But at least you know you have an assay that can give you a signal. And so far, in these vaccines, other than rubella--I mean, I guess it's going to take developing a database to see if this kind of study gives a signal.

MS. BENNETT [In Audience]: I actually want to make a comment about that lady's comment. Sorry, I'm Jillian Bennett, from Australia.

I was a little bit surprised. Because what we're doing in our conventional toxicology studies that we spoke about yesterday is, we're not actually trying to target a maximum lethal dose, or anything like that, because we've recognized that they're vaccines, and we're not trying to induce intentionally a toxic effect. So we put in a dose that we think gives us a margin of safety. And in terms of repro-tox, I actually took it from the same sort of perspective.

In terms of the guidance, I think that I actually have to say, in terms of mapping out our product development program, I found it really helpful to have something additional to the ICH guidance. Because it gives us some perspective to think about with respect to vaccines.

It probably would be helpful if we separated out those vaccines that were intended for women to be vaccinated during pregnancy, versus those who may be unintentionally vaccinated. And I think that would actually bring some clarity then to sponsors, in terms of their understanding of what's required.

I think the other thing is that, in terms of the category of the vaccine, probably vaccines that are recombinant, sub-unit vaccines, adjuvanted with something like alum, you know, people are probably--We have a long history of use of alum but, you know, there is some speculation about the safety of that. But they are antigens that are naturally expressed during infection.

And so I think the epidemiology and understanding of the disease and the sequelae of having the disease are also very useful in terms of what we may want to incorporate. But again, I think that's probably defined quite well in the guideline.

I think from my own company's perspective, where we have a novel adjuvant and it is something that we don't have a lot of experience with, I think it would be immoral if we actually didn't make some sort of attempt to understand the developmental toxicity of that. And perhaps, if we do give a rabbit a 15-fold-high human dose, it might actually also be useful to give the equivalent human dose on a milligram-per-kilogram basis, just to give us an understanding of what the background level is versus an extreme level. Because in our normal animal models where we set our dosing, we've probably given them--you know, almost tried to mimic what a human dose would be. Thank you.

DR. VAN DER LAAN: Okay. Thank you.

I think, the last question.

PARTICIPANT [In Audience]: Okay.

DR. VAN DER LAAN: It's five o'clock.

PARTICIPANT [In Audience]: Oh, okay, very quickly then. We've been speaking a lot about IgG and trans-placental transfer. And when we address, though, working with live virus, that then becomes the concern about the transfer of the virus, in fact, across the placenta. And there are very rare reports of human neonates and IgM. And therefore, the conclusion being that the human neonate probably did see the virus as the result of immunization of the mother with a live virus.

Not being an immunologist, please deal with my technical question here. Would it be technically feasible to think, okay, allow the pregnancy of the dam to go forward and either the pups or the kits, whichever species you're using--Would it be feasible then to measure, given the differences in the immune response? This is my question, though. Could we have a surrogate marker, such as the rare report, as we see, of IgM in human neonate? Is that just not really possible?

DR. VAN DER LAAN: Are there technical persons in the audience who can answer this question?

[No Response.]

DR. VAN DER LAAN: On the panel? No. We have to think about it.

[Simultaneous Discussion.]

DR. VAN DER LAAN: We don't know.

MS. HOLME [In Audience]: Risa Holme [ph], from GlaxoSmithKline.

We have had a live viral vaccine where we've evaluated the ability of the virus to cross the placenta. And we evaluated the sort of standard repro development end points. And since we did PCR on a significant number of pups and we didn't see any developmental tox, we felt that it was adequate to stop there. So we have had experience of actually doing PCR in mice studies following a live viral vaccine.

DR. VAN DER LAAN: Okay. Thanks.

Thanks for the audience for this discussion in this last hour.

I guess, to Marion or Mercedes.

DR. SERABIAN: I'd like to thank everyone for coming and staying. I'm not sure, per se, consensus was reached today on certain items; but certainly, some stimulating conversation, and a lot of issues for us to take back and think about.

Do you want to add anything?

DR. GRUBER: I thank everybody for coming to this workshop and participating in the discussion. That was very helpful. And thank you very much again. 'Bye.

[Whereupon, the workshop was adjourned.]

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