

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES

Meeting of:

SECRETARY'S ADVISORY COMMITTEE

ON

XENOTRANSPLANTATION

July 2, 2001

Holiday Inn
Bethesda, Maryland

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TABLE OF CONTENTS

	<u>Page</u>
Opening Remarks - Harold Vanderpool	1
Conflict of Interest Statement - Mary Groesch	4
Introduction to Cross-Species Transmission of Infectious Agents	
Viral and Host Determinants of Infection and Disease - Jon Allan	5
Global Experience with Cross-Species Infections: Lessons for Xenotransplantation - Brian Mahy	11
Discussion	18
Infectious Agent Transmission in the Allograft Setting	
Infections After Allograft: Lessons Learned - Marian Michaels	23
Xenotransplantation Source Animals and Products: Minimizing the Risk of Transmission of Infectious Agents	
FDA Requirements and Recommendations for Xenotransplantation Animal Sources and Products - Eda Bloom	28
Animal Husbandry Techniques in Swine to Minimize the Risk of Transmission of Infectious Agents - Michael Swindle	36
Lessons Learned About Viral Screening From Other Biologics - Anthony Lubiniecki	39
Porcine Infectious Agents: Implications for Xenotransplantation	
Viral Safety Issues for Xenotransplantation - Clive Patience	45
<i>In Vivo</i> Infection Studies with Porcine Endogenous Retrovirus: Implications for Clinical Trials - David Onions	51
Public Comment	60
Retrospective Studies in Patients to Detect PERV Transmission and Pathogenicity - Dan Salomon	64
Other Porcine Infectious Agents of Concern in Xenotransplantation - Barbara Potts	71

COMMITTEE MEMBERS PRESENT:

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Alan H. Berger, MBA, CPA, Animal Protection Institute, Sacramento, CA

Bradley H. Collins, PhD, Duke University, Durham, NC

Catherine Crone, MD, INOVA Fairfax Hospital, Falls Church, VA [present on Day 2 only]

James Finn, Newport, RI

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EXECUTIVE DIRECTOR:

Mary E. Groesch, PhD, Office of Science Policy, Office of the Director, NIH

EX OFFICIO COMMITTEE MEMBERS PRESENT:

CENTERS FOR DISEASE CONTROL AND PREVENTION

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PROCEEDINGS

(8:45 a.m.)

Agenda Item: Opening Remarks.

DR. VANDERPOOL: Good morning. Let's go around the table and introduce ourselves. I am Harold Vanderpool, chair of the committee.

DR. LUBINIECKI: I am Tony Lubiniecki. I am vice president of biopharmaceutical development for Glaxo Smith Kline. I spent the last several decades working on development of manufacturing processing for recombinant products, and also methods to assure the viral safety of these products.

DR. MICHAELS: Marian Michaels. I am in the division of pediatric infectious diseases at Children's Hospital in Pittsburgh.

DR. ALLAN: I am Jon Allen from the Southwestern Foundation for Biomedical Research. I am virologist and I work with simian retroviruses.

DR. SYKES: I am Megan Sykes. I am a researcher in transplantation immunology, including xenotransplantation. I am at Mass General Hospital and Harvard Medical School.

DR. GROESCH: I am Mary Groesch. I am executive director of the committee and I am also a member of the National Institutes of Health Office of Biotechnology Activities.

DR. VANDERPOOL: A word about my own work at the University of Texas Medical Branch at Galveston. For a long time I have been involved in issues of biologics in theory, and innovative therapies, and have worked a long time on issues related to xenotransplantation and ethical issues pertaining to those. Also, I am a historian of medicine, and recently have worked in some detail on the study of xenotransplantation, beginning with the transfusion of man's blood into mentally unstable patients in the United Kingdom in the 17th Century. I will not burden you with information about that today from there throughout history into the 20th Century and the present time.

DR. KASLOW: I am Richard Kaslow. I am at the University of Alabama at Birmingham, in infectious disease epidemiology primarily, but focusing on HIV infection and other host factors.

DR. SALOMON: I am Dan Salomon. I am a researcher in the department of molecular and experimental medicine at Scripps Research Institute. My interests are in cell transplantation, allotransplantation, gene therapy, tissue engineering and xenotransplantation.

MR. BERGER: My name is Alan Berger. I am the executive director of the Animal Protection Institute. We are a non-profit advocacy organization that is headquartered in Northern California.

MS. SHAPIRO: I am Robyn Shapiro and I am professor of bioethics at the Medical College of Wisconsin and the director of the bioethics center at that medical college.

DR. SWINDLE: I am Michael Swindle. I am professor and chair of comparative medicine at the Medical University of South Carolina. I am a veterinarian and am interested in swine.

DR. RUSSOW: I am Lilly-Marlene Russow. I am a professor of philosophy at Purdue University, where I teach ethics in both the philosophy department and the School of Medicine.

DR. COLLINS: I am Brad Collins, transplant surgeon at Duke University Medical Center.

MS. KING: I am Karren King. I am a social worker with experience in both dialysis and kidney transplant. I work in Kansas City, Missouri.

DR. SCHECKLER: I am Bill Scheckler. I am a professor of family medicine at the University of Wisconsin Medical School in Madison, and also an epidemiologist at St. Mary's Hospital. I am here as a representative of the CDC's health care and infection control practices advisory committee.

DR. MAHY: I am Brian Mahy. I am a senior scientific advisor at CDC.

DR. POTTS: Barbara Potts, I am director of QC Biology at Genentech.

[Other introductions made around perimeter of room off microphone.]

DR. VANDERPOOL: Thank you. The last meeting, which was held four-and-a-half months ago, was a meeting in which we discussed and discovered a great deal about xenotransplantation in terms of basic education. Then we spent a good portion of the meeting talking about structuring what the various issues are -- social, psychological, ethical, scientific. Now, today we are going to be focusing this day on infectious disease issues. We are most pleased to have a number of the committee members make several of these presentations. Others who have been invited are sitting at the Logan Airport in Boston, wanting to travel to Washington. Dr. Groesch will tell us about certain revisions in the timing of the program, because of those delays by Clive Patience and David Onions.

Thanks in part to email exchanges between various committee members after the last meeting, we all agreed that one of the truly pressing sets of issues that we need to deal with are concerns over infectious disease. These issues are, indeed, gatekeeper concerns, whether we should go forward or not, to put it in the most stark contrast. Our challenge is to assess how serious these infectious disease issues are, both in terms of cellular xenotransplants and organ xenotransplants. One of the innovative and important things that Dr. Groesch has brought to our attention is that we need to be able to assess these risks of xenotransplantation in light of the risks of allotransplantation which often are not given due attention.

So, we are going to talk about these issues. Then tomorrow we are going to spend an entire session on what the import of these issues is for clinical trials, for consent forms, for the possible need of further research prior to the onset of certain clinical trials, to the adequacy of the present federal guidelines. We need to talk about whether these issues are serious enough to call for a third party consent of close contacts or maybe even communities. We need to say, well, to think about, if these are serious enough, what back-up plans do we have in case infectious disease is discovered in xenotransplant recipients.

Now, I want to give a little perspective with respect to the history of these infectious disease concerns, which at least situates this in time and place. Part of what I will say and part of what I will recommend briefly is indebted to a long and certainly insightful discussion with Dr. Louisa Chapman very recently.

Prior to the 1990s -- certainly we see this in the 1960s -- the infectious disease issues were not of particular concern over xenotransplantation. They were viewed more or less as similar to allotransplants. When the Institute of Medicine met at the beginning of 1995 and produced its report in 1996, that report on which a number of committee members here contributed, talked about these issues as serious. Shortly after that report was produced in 1996, because of discoveries about the endogenous retroviruses curve, these issues became very serious, to the point that the FDA placed a hold on clinical trials and cellular

transplants until more sensitive assays could be developed and until certain studies on patients who had received xenotransplants could be explored with respect to infectivity.

That was a pretty momentous time. It is not very many years ago, but it was a rather momentous time. Certain persons across the United States – Fritz Bach and others – called for a ban on clinical research because of these worries over infectious disease. Organizations were developed, which began to oppose xenotransplantation because of infectious disease potential. I have in mind the Campaign for Responsible Transplantation executive director, which is Alix Fano.

Now, some of these concerns put a damper on the degree of excitement over xenotransplantation that existed at the time. Some of this lessened enthusiasm is reflected in official reports, such as the United Kingdom's xenotransplant interim regulatory authority. Reflected also in some of these concerns is the degree to which some industries have withdrawn from xenotransplantation research, because of some of these concerns, possibly related to litigation. The question really to keep in the forefront of our discussion is, are these concerns warranted. What level of concern is warranted, or why or why not, or are such concerns warranted, whether serious or not so serious, what does the data actually say.

At the present time, there is this almost disjuncture between people who are terribly worried about infectious disease, and others, such as Jonathan Stoye – I hope I am pronouncing his name correctly -- who worked with Robin Weiss, who called PERV -- porcine endogenous retrovirus -- wimps and he said that xenotransplanted pig organs, concerns over this risk regarding this are virtually non-existent. The question is, what really is the situation. I think one of the challenges of this committee is to seek to clarify what these concerns are, to be very clear about it. We probably have some significant responsibilities to the public in terms of clarifying of undue alarm on the one hand, and perhaps a disregard, or at least a lack of regard, for the possible seriousness of some of these issues. One of our real challenges is to think through what these issues are, become clear about these, so that we can move on to other issues.

Now, tomorrow we will also begin to discuss and formulate plans for future subjects of the SACX committee. I want to encourage each of the SACX members to begin thinking about, okay, what do we need to discuss next and then next beyond that. These issues are so important in terms of what we should discuss, that it depends on what the function and service of this committee will be or will not be, and what do we represent. I think we need to step back occasionally and ask what our committee is about. I mean, we know what our challenges are, what our constitution says we should do. In specific terms, are we primarily a public forum or are we primarily a group that should respond to the questions put to us by those federal agencies? Are we to review particular protocols? Are we to recommend health policy initiatives at a governmental level? I suspect we are to do all of these, but we need to choose which to assume first.

Just for the sake of getting our thinking going in the direction of what our future meetings might be, I want to give a brief, tentative take on what this direction could be. All of you who know me know that my tentative takes are, indeed, tentative. I think that from my perspective our most critical mandate is to think about xenotransplantation with respect for better health for American citizens and beyond the United States. We really need to cut through whatever misinformation that might be out there and ask what is the promise of xenotransplantation both on the cellular and on the organ level, and to keep ever in mind that we are interested in promoting better health. Whether industry does or does not see xenotransplantation as profitable enough to pursue research initiatives, if we view, in our discussions, that these xenotransplantations can indeed be of great promise for human health, then we need to take steps to encourage its development.

Maybe our next meeting, or a meeting very soon, should be to do a couple of things. Review all present and proposed clinical trials. That may take a closed meeting or two, and we need to talk about that. So, I think we need to have a good handle on what the present and proposed clinical trials are. I think we also need to have a good solid grasp of what the alternatives to xenotransplantation are. How close are we to developing human organs through stem cell research? That is going to be a hard judgement call. Are we eight years away, 10 years away, 15, 25 years away? If we are pretty far away, we have patients who are suffering and pining away on waiting lists, hoping for some rescue. In that sense, xenotransplanted organs would be the best interim measure that we could encourage if other issues, including, of course, infectious disease are not of major concern. Where else does xenotransplantation hold great promise? Might it be the best option with respect to alleviating the scourge of diabetes? Might it not be the best option for being able to rectify the terrible effects of spinal injuries?

We have seen some disappointing results regarding Diacrin's study of Parkinson's disease patients. Dr. Chapman has rightly raised the question as to whether the small number of patients in those clinical trials gives us a good read on the success, or lack of it, in terms of piglet neuron cells implanted in Parkinson's disease patients. We need to look carefully at what the real promises are. We can't do that, in my judgement, without knowing where the alternatives are in stem cell research, on the one hand, and certainly in terms of mechanical modalities on the other. I just present this as a possibility to encourage us to begin thinking about what the pressing issues are for us as we move forward, and as we prepare -- I should say as Dr. Groesch prepares us -- for our future meetings.

With those comments in mind and with those commissions in mind, let's move to the program of the day. Dr. Groesch first has comments. Then we are happy to have Dick Kaslow as the facilitator of the various presentations that will be made by committee members and others. Thanks very much.

Agenda Item: Conflict of Interest Statement.

DR. GROESCH: I have a very brief statement to make about rules of conduct and conflict of interest. As members of this federal advisory committee, you are special government employees and are therefore subject to rules of conduct that apply to government employees. These rules and regulations are explained in a report entitled Standards of Ethical Conduct for Employees of the Executive Branch, and you each received a copy of this document when you were appointed to the committee.

At every meeting, in addition to reminding you about the importance of following the ethics rules, we always like to review the steps that we take, and ask you to take, to ensure that any conflicts of interest between your public responsibilities and your private interests and activities are identified and addressed. As you know, before every meeting, you provide us with information about your personal, professional and financial interests. We use this information as the basis for assessing whether you have any real potential or apparent conflicts of interest that could compromise your ability to be objective in giving advice during committee meetings. If such conflicts are identified, we either issue a waiver or recuse you from a particular portion of the meeting.

We usually waive conflicts of interest for general matters, because we believe your ability to be objective will not be affected by your interest in such matters. We also rely to a great degree on you, to be attentive during our meetings to the possibility that an issue arises that could affect, or even appear to affect, your interests in a specific way. If this happens, then we ask you to recuse yourself from the discussion. If you have any questions about the rules of discussion or conflict of interest, our committee management officer, Ms. Mary Nuss, is here this morning, and she will be happy to talk to you at the first break.

As many of you experienced first hand in trying to get here, there were some very nasty storms that have moved through not only our area, but I think the entire coast and even the midwest. As a result, a couple of our speakers -- David Onions and Clive Patience – hopefully will make it here this afternoon. I think what we are going to do is actually switch two sections of our meeting. We will have our first session on introduction to cross-species transmission of infectious agents, and our second session on infectious agent transmission in the allotransplantation setting. Then we are going to flip the next two sessions. The third session will be on xenotransplantation, source animals and products. That will be flipped with the porcine infectious agent speakers. Hopefully, by the time we get to that, we will actually have the speakers here. We will just have to wing it a bit in terms of keeping on schedule.

DR. VANDERPOOL: Okay, Dr. Kaslow.

DR. KASLOW: In typical government committee fashion, we are starting about a half an hour late already. I think we should begin promptly with introduction to cross species transmission of infectious diseases. First, Dr. Jon Allan from the Southwestern Foundation will talk to us about viral and host determinants of infection. Jon?

Agenda Item: Introduction to Cross-species Transmission of Infectious Agents. Viral and Host Determinants of Infection and Disease.

DR. ALLAN: I have sort of been given the task of giving an overview on what things to think about when you are looking at viruses and host determinants of infections and disease, and how that relates to the xenotransplant setting. There are a lot of different viruses and they all have different strategies, and the host has to deal with each one of those viruses, sometimes in very peculiar ways. That dance is really what ultimately determines whether or not one sees infection and/or disease. So, I am just going to give you generalities. There will be others who will talk about specific infections. Primarily, I will just give you a sense of what areas we need to consider in the transplant setting. I thought I would introduce the terminologies. Sometimes people throw out these terms and especially zoonosis, and maybe some of you aren't that familiar with some of these terminologies.

Zoonosis is really a naturally occurring infection in animals that may be transmissible to humans, with or without human/human transmission. So, when you think about xenotransplantation, people talk about zoonosis. Really, what we are concerned about is a second definition, which is emerging infectious disease, and that is, does the virus establish itself in the human population. It is just as zoonosis dead ends in a particular host, it really stays a risk to the patient, but really what we are most concerned about is the risk to the population. It is sometimes difficult to sort out when zoonosis becomes an emerging infectious disease. Really, HIV began as a zoonosis, but I wouldn't consider it a zoonosis at this point. It is really a human disease, a human infection disease.

Now, there are a lot of different steps that one can look at in terms of the risk. This is just a simple schematic of what points do we have to consider in terms of infection and disease. Obviously, there is a pig organ, or previously a baboon organ, and the first thing we want to consider is, do they have viruses, and you can screen for those, except for the unknown viruses. For the most part, with the known viruses, you can probably screen the herd, find out what is there, eliminate most of those. In some cases, you may not be able to eliminate all the viral infections, and we know about PERV so far, and then you look to see whether the virus can replicate in the human host. It may be confined to the tissues, but it still can replicate. So, it is difficult to determine whether or not the virus is replicating in the pig tissue or actually has been transferred to human cells and is replicated in human cells.

The next step is, does it infect human cells? That, you can do *in vitro* studies, use human cells in tissue culture to look at that. Then, does it actually replicate in a human host? Does it reproduce itself, does it express itself? Do you get a viremia associated with the infection if, in fact, the human cells get infected? Obviously, if you get a viremia, what is the possibility of a transmission? So, we make predictions about, if a virus is expressed in a human host, whether it is going to be transmissible to another person. This can be very difficult to determine retrospectively, before the transplants occur.

Obviously, the last question we want to ask is, you know, can we make predictions about disease based on what we know on any of the model systems and on the virus/host interactions? This is obviously another area that can be very difficult to sort out, and that is pretty much what I am going to address here.

The routes of entry, what I am going to do is just take you really through what viruses specifically -- not the actual viruses, but the general types of viruses that we really have to worry about in a transplant setting. Now, viruses have all kinds of ways of penetrating a human host. There are natural barriers and a lot of viruses can infect fecal orally, respiratory tract infections, urogenital tract infections. Some penetrate the skin. There are also mosquito vectors. Most of those viruses, I think, are not relevant to the transplant setting. Really, the viruses that are most relevant to the transplant setting, at least, are the ones that are pretty much blood borne pathogens or have the potential to be sexually transmitted diseases.

The reason I say that is, environmental viruses -- in other words, fecal oral or respiratory tract infections -- we have been in contact with pigs and other animals, so that we probably would have been exposed over the thousands of years. We are really thinking about viruses that wouldn't normally have the potential of infecting a human host. That is why we consider blood borne pathogens and sexually transmitted diseases, because those are not typical viruses that can be transmissible to humans.

To really look at some of the determinants that one needs to consider in terms of whether the virus is infectious to the human cell -- and that is the first step as I mentioned earlier -- we have to look at the life cycle of a virus. This particular virus is HIV, because I work with simian retroviruses and HIV. It is basically just a template. You can put any other virus on it and there are general considerations here.

One is the initial interaction between the virus and the host cell. That usually comes through a receptor. That can actually determine tropism. It can determine host range itself. So, if the virus is promiscuous and uses a conserved region of a cell surface protein, then it may have a wider host range. There are other viruses that bind to very specific sites on receptors that are less conserved among different species. You can actually make predictions based on tissue cultures and knowing the properties of the virus, whether or not a particular virus would be able to bind and enter a human cell. There are some viruses that do and some viruses that don't. There are multiple receptors. Not only do you consider a single receptor but, in the case of HIV and several other viruses, there are multiple receptors that all have to be there and all have to be conserved for a virus to jump from an animal to a human.

There are a lot of barriers here in terms of a virus coming from one host and getting into another, once it penetrates, whether it gets endocytosed or whether it actually fuses through the membrane. Then, for the virus to replicate, it in most cases has to use the host cell machinery. In some cases, the conservation between those regulatory proteins that are necessary for the virus to replicate -- and these are cellular regulatory proteins -- the degree of conservation will also dictate whether or not an animal virus will replicate in the human host. So, even though the virus gets into the cell, it still has to replicate, and there are different viruses that use different cellular proteins to replicate, and some are more complex than others. So, there is another barrier there in terms of cross-species transmission, which is conservation of these cellular regulatory proteins, before the virus can actually exit the cell. These are just some of the

things to consider.

The next level to consider is whether the virus is an acute infection or whether it is a persistent infection. Typically, the acute infections we can deal with in general. What happens with most acute infections is, they are cleared. If you think about cold viruses or flu or some of these other viruses, they don't persist. In other words, you get infection, you get sick, the immune response kicks in, you clear the infection and you no longer have it. Obviously, in a xenotransplant setting, if you are looking at particular animal herds -- pigs, whatever -- if you isolate them, you can probably get rid of most of the acute infections that are a potential risk to humans. There are certain attributes of acute versus persistent infections. In the persistent infections, what I am saying here is, you get infected, you don't clear the infection. It may be a lifelong infection, and this is what we are most concerned about. So, we can clear the table of a lot of acute viral diseases and focus more on the persistent ones.

There are some sort of characteristics about acute versus persistent. These are gross generalities. Some viruses don't fit these categories. The acute infections are often disease associated. They usually have high mutation rates. They are usually RNA viruses, but not always. They typically have a wider host range. They usually can infect multiple species. The reason for this is because in acute infections, in order for them to survive, what they have to do is keep jumping from host to host to host. So, they have to have a high population density so that they can continue to circulate in the animal or human host. They don't usually show as much co-speciation with the host. That is, there is not this sort of species-specific virus situation that you generally have with persistent viral infections. That also goes to the xenotransplant setting, too. Persistent infections tend to be highly species specific, but not always. What that means is that the farther away you get from humans, or you deal with pigs to humans, most of the viruses that are persistent infections don't normally, or it is much more difficult, for them to go from pigs to humans or from any animal species to another.

Transmission of acute infections tend to be horizontal whereas, in persistent infections, they can be maternal/infant, they can be sexually transmitted. Again, I said, persistent infections are less dependent on host population density because the virus strategy to survive, if it is persistent, it can sit around and wait until it finds another host to infect. The persistent infections, obviously, are the most dangerous in this particular case. So, we have cleared the table of mostly acute infections.

Let's go to persistent infections. This is an example. This isn't all of them. Again, there are lots of different virus types, adenoviruses, herpes viruses, hepatitis viruses. Again, they have different strategies and tissues affected based, both, on the tissue receptors that they bind to and regulatory proteins.

The adenoviruses are persistent but they generally don't have a long-term outcome. They are just acute disease and then they persist. So, they can sit and wait for a new host. The adenoviruses tend to be highly species specific. Usually human viruses only infect humans, but they can also infect chimps and, in some cases, baboons. Herpes viruses are also species specific for the most part. They can jump, in some cases, between monkeys and humans but they tend also to be something of consideration in the transplant setting, but of less concern. Hepatitis viruses are both RNA and DNA viruses. They usually are restricted to hepatocytes, again, persistent infections. These also tend to be highly species specific, most of them. Hepatitis E isn't, but I don't believe that is a persistent infection anyway. Most of the hepatitis viruses, you can inoculate them in a chimp and get infection, and that is probably where it ends. Papilloma virus is also highly species specific, except for bovine papilloma virus. Human can get that. For the most part, it is restricted to humans. Polyoma viruses, these are also species specific. There have been cases where SV40 has been found in people, and we are going to hear about that later.

So, if we just jump down the list, we end up with HIV and HTLV, which are basically both retroviruses. These are, for the most part, species specific as well. These are complex retroviruses and, therefore, they are generally going to be more species specific. Again, they go from primates to humans. So, a lot of these intermingle between primates and humans but not other species. So, if you just look at the face value, we are basically dealing with retroviruses. There are a few other [word missing] viruses which we know very little about, but those are also highly species specific. So, with persistent infections, we are really pretty much down to retroviruses and maybe a couple other ones. We are not really clear about circaviruses and a couple of others. So, when we think about xenotransplantation, and people talk about all these different viruses that are out there that could be infecting people, the environment and whatever, keep in mind that most of them are unlikely to infect humans.

If you look at viral replication patterns in terms of the zoonoses, there again is acute infection. It gets cleared right away. This is an example of HIV. There are other viruses that also do this. You get an acute viremia and then the host immune response kicks in and essentially the viruses drop to very low levels. Then at some later time -- and we are not really even clear about why this happens -- but you get a resurgence of the virus being expressed and you get clinical disease. This is sort of a template for concerns in the transplant setting. You have an acute infection followed by just a lot of viremia.

Something I have just included here, this is mostly hypothetical, but it is you get an infection from an animal virus. It replicates very poorly over time and, through adaptation and selection, you may get viremia. This may occur within the individual or a population. So, this doesn't necessarily have to happen with an individual, but it could happen in a population. So, these are really things to keep in mind.

Mechanism associated with the establishment of infection, people seem to talk about mutations, what happens if the virus mutates and all of a sudden it is really an important pathogen in humans. Really, when you think about it, viruses are mutating constantly, regardless of what host they are in. When a new virus gets into -- let's say an animal virus gets into a human. It essentially has a new ecological niche. I mean, it has never seen a human cell before. What does that virus want to do? It wants to survive. So, it is going to try to adapt itself to that host to replicate. That is what it is going to do. It is going to try everything possible to do that. It is just like if you took -- there are so many instances where, if you take biological organisms from around the world and introduce them to the United States, and you get kudzu or you get all these different problems with foreign invaders. You can look at this as a similar situation.

Also, when you think about mutations and adaptations, you can think about it as punctuated equilibrium, which means that it is not a gradual evolution of a virus in terms of its adaptation to the host. Usually, when you change the environment dramatically, the virus is going to change itself dramatically. So, you can get hypermutations occurring as it finds its way into the human host. We also know that, in the case of simian models for AIDS, if you take a virus and you passage it very rapidly between monkeys, you heat the virus up and you make it much more pathogenic, and I will talk about pathogenicity in a moment.

The *in vitro* rate of replication may also predict host pathogenicity. If a virus replicates very well in the human transplant recipient, it is more likely, at least to be pathogenic. It may not be. It is very difficult to predict. It might also increase the chances of transmission. So, there are a lot of evolutionary forces on new human infections. If you are dealing with retroviruses, the enzyme they use to replicate themselves, there is no proofreading capability, so you get a higher mutational rate occurring there. There is positive selection that occurs in immune systems, driving variation to occur, and that may allow for greater adaptation. You get an evolutionary master consensus sequence, which may not be the same

as the host, and that is what I mean by new ecological niche. Also, recognize that an inoculum, whether it is an organ or it is cells that are introduced, actually represents a swarm of viral veterans. It is not just one virus.

What comes out may not be what goes in. This is just an example of what could happen. If you introduce a virus here, as it adapts itself to a human host, you may get a consensus sequence that looks very different than the original. This is a very common situation. Even though you start with one virus, you could end up with a whole number of variants that are tissue specific. They may change tropism. You also have to look at the immune response to viral infection. Obviously, I mentioned the primary role of clearance in acute infections, be it neutralizing antibodies, cellular immune responses, innate immunity, that happens very easily. Another thing you have to consider is what I call exacerbation and causality, which is that in some instances, viruses which are infectious may not be pathogenic, especially in -- here are a couple of cases here. SIV is one of them.

There is another virus in mice that, if you put it into newborn mice so they are essentially tolerized to the virus, they don't get sick, because they don't mount an immune response. Sometimes the immune response can be deleterious to the host. So, even though it is important for the host to clear the infection through the immune response, it could also exacerbate the infection. One way to call that is innocent bystander effect. It just sort of blasts a whole bunch of cells, including infected cells. You can also have replication in immunologically privileged sites, which are barriers to immune cell responses, such as in the neurons or reproductive cells. You can have natural heterophilic antibodies, which you may hear about later, which are antibodies in humans which are actually very beneficial in terms of eliminating animal retroviruses at least.

One of the issues here is that if you remove the alpha gal protein from pig cells, you are going to remove them from the viruses. That could actually increase the risk of a pig retrovirus in humans. You could have immunologic tolerance which, whether it is induced or natural tolerance, may allow for unregulated viral replication and spread of infection. So, that is something that has to be considered, too. There is a whole list of other things you consider, the age-related effects, immunosuppression, positive selection, as I mentioned before, the super-antigens. There is a whole dance that the virus does with the host. The virus is trying to invade the host immune system and the host is trying to kill the virus. Each has its own strategy in order to do that.

When we look at pathologic consequences of infection, you know, this is the obvious thing. You can get infection in both natural and unnatural host and there are plenty of examples of that. You can get no disease in the natural host, but then you get disease in the unnatural host. Obviously, that is the thing that we are worried about in the transplant setting, and there are plenty of examples of that. There is disease in the natural host but not the unnatural host. In other words, it could be aboard but it doesn't replicate very well in the human host. You can get no disease in either host, and an example of that is simian foamy virus, which is another retrovirus.

Some of the things to consider, when you think about pathogenicity of a virus, it is very difficult to predict whether a virus that is found in a natural host will be pathogenic or cause disease in an unnatural host. This is what I study, which is the simian immunodeficiency virus. In the natural host, it doesn't cause any disease. It is transmitted very similarly to HIV in humans. It infects the same cell types. It uses the same receptors. You get a high viremia in the natural host, but you don't get disease. The other interesting thing is the immune system is basically quiescent. There is very little immune response to viral infection in the natural host, whereas in the unnatural host, you have a lot of immune response, but you obviously get disease. You get AIDS. So, what this basically says is it is very difficult to predict

what is going to happen if the virus gets into the human host, in terms of the pathogenicity of the virus.

Here is an example of simian foamy virus. These are human cells, fibroblasts, infected with the simian foamy virus. You get a large amount of cytopathic effects. Does that predict disease in humans? No, it doesn't. Sometimes the cytopathicity of a virus doesn't necessarily correlate with its ability to cause disease in a host. This is a leukemic cell in baboons. They are infected with a retrovirus called STLV. It is very similar to HTLV. So, not only can retroviruses kill cells, they can also transform cells and immortalize cells.

Another issue here is, in terms of pathogenicity, this is a simian immunodeficiency virus infection in macaques. They use a molecular clone. What happens is that, over time, the animal gets sick. If they take the viruses out at the different time points and then use them to infect other monkeys, what they find is that the virions that come out later on are much more pathogenic than the other ones. There is an evolution toward pathogenicity, at least in an individual. This is something we are concerned about, obviously, in a population where you get enhanced pathogenicity. When you think of pathogenic consequences of retroviral infections, you have simple retroviruses and complex ones. The simple ones basically are weakly oncogenic. They don't have a bunch of accessory genes that are used to replicate. They basically have gag, pol and env, which are basically structural proteins to replicate.

The complex retroviruses are more difficult to deal with because they can be also oncogenic or cytopathic. There are many ways in which retroviruses can cause cancers. This is obviously something we are concerned about when you think about PERV or some of these other retroviral infections that may jump into humans. Retroviruses have lots of strategies in order to induce cancers. These cancers can be long-term cancers. In other words, the infection happens and it may be decades before one sees any disease associated with these infections. You can have acute tumorigenicity where the retrovirus actually transduces a human oncogene, and those tend to be more acute infections, probably not as important in this situation. You can get recombination -- people talk about this -- between exogenous and endogenous retroviruses. That can change tropism, that can change pathogenicity. Retroviruses produce not just tumors and AIDS, but lots of other types of diseases that you have to consider. Last, the insertion of the germ cells may result in endogenization. This is a small risk but it is something that I think everybody needs to consider.

If the retrovirus can get into the germ line and insert itself, it could actually change evolution in humans. This just shows how the retroviruses do that. This is through recombination with an endogenous envelope. Like I said, it can change tropism, it can change pathogenicity. It can actually pick up an oncogene, or it can just insert itself, like the simple retroviruses. As it inserts itself into the human genome, it can be upstream from a protooncogene and, therefore, cause tumors in the long run.

Just to finish up, the first question is, can we make prediction about infection of human recipients. That is actually not that easy to do either. If you base them solely on *in vitro* infectivity data, you would probably eliminate a lot of infections, because there are viruses that infect human cells in tissue culture, but they don't apparently infect humans *in vivo*. So, it is sometimes hard to tell.

So, prior human trials provide some measure of risk assessment. You will hear about those, but they may not reflect the present situation. So, the type of organ, especially the duration of the transplant, the exposure, and the level of immunosuppression may affect whether or not an infection actually occurs. It is even more difficult to predict pathogenic risk in humans based on the infection in the natural host, as I mentioned earlier, and it is obviously precarious. You have a long latency period and you have adaptation. These are things you really need to consider. Predictions are also difficult based on early

events if infection does occur. So, if you see infection and the patient is not sick, that doesn't mean that the virus isn't pathogenic. Predictions about transmission, I think, are probably even more difficult to make. You are not going to know whether something is transmissible or not until it actually happens. That may be related to the level of viremia, route of transmission, and the preventive measures that you use.

There are also host factors that contribute to the amount of infections that may enhance the risk of establishing a new public health problem, such as immunosuppression, age of the recipient and underlying infections, all of which may exacerbate an infection, if it does occur. This is a plug, but suitable animal models to determine infection and disease are obviously warranted in any situation where you are looking at potentially a new infection in humans.

So, I think I will stop there, and if there are any questions, I would be happy to take them.

[Applause.]

DR. VANDERPOOL: Thank you, John. We are still running late. If there is a brief question of clarification only, I will take that, but I think we should go on to the next speaker, who is Dr. Brian Mahy from the Centers for Disease Control, who will give us some insight into the global experience with cross species infections, lessons for xenotransplantation.

Agenda Item: Global experience with Cross-Species Infectious: Lessons for Xenotransplantation.

DR. MAHY: Thank you very much indeed. I would like, in the next 30 minutes, to give you just an overview of some infectious cross-species events, but it will be confined really to viruses. I was asked, really, to talk about the experience in terms of virus infections. One of the things that is very clear, I think, and very obvious is that there has been quite a dramatic improvement in our ability to detect and to investigate infectious diseases.

This just gives a very brief series of examples globally of events that have occurred in terms of new viruses appearing. You will see that most of these occurred in the 1990s or even since 2000. They range in different types, for example, from West Nile virus which really got taken from the Middle East and is well established on the east coast of America. There were situations that were completely unexpected, like the appearance of Nipah virus, which I will talk about a little bit, because it is important in terms of porcine infection, and Hendra virus occurring in horses. There are various unexpected infections, the influenza that occurred in 1997. Then we have this continuing problem with cross feeding infections from species we don't even know the origin of. Ebola virus, most recently, in 2000 we had a major epidemic of Ebola virus.

So, essentially there are many of these, and you may wonder why these infections are being recognized. I think it is probably because the Institute of Medicine drew attention to this problem in 1992. We have had increased opportunity, I think, for surveillance that we didn't have before. Our surveillance for flu viruses, especially in the China region, is much better than it used to be, and also we have much better techniques, much better methods for looking at different viruses, so that we can use molecular techniques now to distinguish between viruses.

I would like to start by going back to look at a couple of examples of cross species infections between animals because, in some ways, these are better investigated and have given us a basis on which we can think about cross species from animals into humans. This is a series of examples of events which have

occurred, starting with probably what was the best understood example, which is still not completely understood, but the movement which occurred of a virus called feline infectious peritonitis from cats into dogs, which occurred in the 1970s. The feline virus had been known for almost 100 years and yet the first evidence that it could move between species was when it moved into mink in the 1940s. Then suddenly, in the 1970s, it moved into dogs and was found around the world. We know now that the movement across species is actually due to changes in the VP2 protein, which is an encapsulated protein of the virus in cats which, once it has these changes, it can affect dogs. So, this shows really that very little change is needed, very small mutations are needed, to change the strain of the virus. I think it is an important lesson which we need to bear in mind. It is a very small single stranded DNA virus, but a relatively small mutation can rapidly change the whole strain. Curiously enough, we now know from recent work that is coming out of Asia, that this dog virus has now gone back into cats, with small cats, and now the smallest isolates from cats are actually viruses that were originally in dogs. So, it went back and forth between species with very small mutational changes.

There are other examples here which I don't want to talk about in detail, but I will simply note that the BSE agent moved from sheep into cattle, which was a major change, which was caused by a change in food rendering in the United Kingdom. There is a dog virus which can affect seals, canine distemper, which has been picked up and has been shown to be exactly the same virus as canine distemper, but in fact, is found in seals. In the same way, canine distemper has also moved into lions in the Serengeti Park, and it has caused disease there. So, these viruses are capable of moving between animal species.

One which is important in terms of pigs is Lelystad virus, which is an arterial virus which causes porcine respiratory and reproduction syndrome, or PRRS. This was first recognized in Europe but soon after recognized in America as a different strain. We don't know too much about this virus, but we do know that it is probably related to a virus of rodents that moved into pigs relatively recently.

I will next talk in some detail about Hendra and Nipah virus, which are largely of importance to humans as well as animals. We know, for example, that fruit bats are a source of many of these viruses. The Hendra virus, which is from Australia, from Brisbane, moved into horses from fruit bats. The Nipah virus also moved from fruit bats into pigs. I will talk about it in detail in relation to human infections.

Then we know another virus here which is one of a large group of new viruses which has been discovered. This one is called Menangle, which is an infection of pigs which causes very serious problems in terms of reproduction. This was first described in the late 1990s. It has been found in at least two humans, who were infected with flu-like illness from these pigs. It is believed, because of antibody studies, that fruit bats actually were the reservoir for the virus. It is a very curious virus because it has not really been repeated in terms of -- I am talking about Menangle virus -- it has not been found extensively elsewhere except in this region of south Australia, in New South Wales, where it was first found in August 1907. In pigs, it causes still births and abortions. We know that in the two humans that develop influenza-like illness, they also had antibodies. So, they definitely got the infection.

This appears to be a Rubio virus, as some of these new viruses are. The Rubio virus is a new genus which has been named, the first virus of which is mumps, a virus of humans. It also includes Newcastle disease, a virus in birds, and a number of other viruses. The diverse strain of these viruses is currently unknown and we need to know far more about these viruses, but they are spread by fruit bats, which is actually distributed fairly worldwide, and certainly from the east, as far as Madagascar, this particular species of fruit bat is found, that hangs in the trees upside down. This picture was taken in Queensland. We also see fruit bats down in Brazil, for example, and Mapuera virus is another virus isolated in Brazil from fruit bats back in the 1970s, which belongs to the same group of viruses.

Now, if we look at the human situation and movement of viruses into humans, the classic example and the best known of that, of course, that happened rather recently is HIV, causing AIDS. You see here that we really don't know too much about why this virus moved from chimpanzees into humans. It is now well known that it is a chimpanzee virus that caused HIV-1 and certainly HIV-2 was caused by movement from a sooty mangabey into human. Up through here, this is not only the possibilities, but certainly contact between monkeys and humans, as in this example of the picture from the left, could cause transmission from bites from animals, let's say. I don't have to remind you, I don't think, here that HIV/AIDS is a global problem, that there are more than 36 million people infected, and in particular regions, such as here in Russia, we have this very dramatic increase in the last few years in the number of AIDS cases. So, this is a major, major zoonosis which has spread worldwide from human to human. That is the critical point, really, is why does a virus, having spread into humans, spread so readily in human populations. We don't really know the answer from HIV, but this is a quote from Cedric Mims from a few years ago in which he said that viral infections derived from animals are not transmitted from humans to humans. So, most infections are not transmitted from human to human, or are transmitted with low frequency.

We have got an exception, which is influenza A, which turns out to not be very surprising, giving that many of the viruses are already human and adapt their transmission between humans. You can actually reassort genes with influenza, the genes that separate into apes are separate segments. So, here you can reassort genes that were human in original origins, with genes from another virus, actually the bird virus, which is the main reservoir of influenza viruses, to produce infections in humans. We all had a tremendous start in 1997 when we realized that influenza could actually go directly from birds to humans and was not going through any intermediate host. The intermediate host has been thought of for a very long time as being critically important in influenza transmission and that it was the pig. It was generally believed that viruses had to undergo some sort of reassortment in the pig and that viruses then went from pigs to humans. We know, for example, in 1918 the influenza virus was very similar to the virus isolated from swine at that time. In actual fact, what we saw in Hong Kong at this time was the direct movement from birds into humans, and all the humans that were infected -- 18 in total -- this shows the epidemic curve, and they had direct contact with chickens. So, there was an isolated case back in May and then there were 17 further cases beginning in November. It was decided in Hong Kong to cull more than a million chickens. This resulted in the disease going away and there have been no further cases, although the viruses of this type have been isolated most recently from chickens yet again. So, there is a continuing problem here in terms of the possibility of the chicken virus moving into humans.

What I would like to do now is to say that this is really rather a special case in which we can do some very, very good molecular biology to understand where this virus came from and how this virus moves. If you look at the origins of the virus, this is the virus that I am talking about, the Hong Kong 1997, we call it, H5N1 virus. We now know that it is really a matter of molecular biology of this virus, in particular, the internal genes, that is to say, genes not coding for the envelope proteins. It turned out to be all identical to those from a virus called H9N2, which is a quail virus. Subsequent to this event, there have been human infections with H9N2, which have not actually been fatal, but there have been quite a number of infections in China and in Hong Kong.

We now believe that these internal genes offer at least one key to the way in which it infects humans. The derivation of the alpha protein probably was from two different viruses, one from Guangdong, and one here, which is a Teal virus, H6N1. Now, the external genes which were transferred here included the hemagglutinin. For a virus to actually cause a fatal infection, we know from studies in birds that generally speaking it requires a cleavage process in the human which allows it to become very infectious. Basically, what this shows is the sequence at the cleavage site, which is sensitive to proteolytic enzymes

such as tripsom, and tripsom-like enzymes in tissues allow the hemagglutinin to be cleaved, and that makes it infectious. If you have a site which is highly susceptible to these enzymes, then it changes the pathogenicity. At this cleavage site here, for example, viruses which are not virulent have only a single arginine residue, which is cleaved by tripsom.

In viruses that are highly pathogenic, things like the Hong Kong virus here, the H5N1 virus, you have lots of arginines and lycines here, which can be cleaved and they can then be essentially cut by a number of different enzymes and the virus can then spread. What he has found in the case of birds is that the virus spreads to the brain and kills the bird because the virus is in the brain. So, these changes are extremely important in terms of the movement and the virulence of these viruses.

I would like to say just a small amount about the BSL4 viruses, viruses which require containment and which are really very deadly and have very high degrees of human fatality. These are cross species infections, although in the case of Ebola, which is probably the most important of these -- this is ebola virus here -- we really don't know the reservoir of the virus. We don't know where it is coming from. So, we can talk about cross species infections, but we don't know where it is coming from in the first place. These are interesting viruses. They have a genome which is about 19 kilobases long, very similar in size to the virus that I am going to talk about in pigs in a moment, the Hendra virus and the Nipah virus. Clearly, we have very high case fatality rates, 53 to 88 percent with a high amount of hemorrhage. We know these viruses can spread from person to person. An infected person can actually spread the virus through skin lesions and other routes into others. These tend to be dramatic outbreaks in which particularly hospital based infections occur to a large degree, and the reservoir is unknown. The only clue we have to the reservoir at the moment, and I think a reason why we have to be very careful in talking about infections and where they are and what they are, this is the mortality of Ebola.

There is this virus here, which is a virus which looks very much like Ebola. It has got the same little crook at the top. It has a very similar size and the same structure of the nuclear capsid. You can't probably see on this slide, but this is actually a virus of thrips. It is a virus from thrips, which infect plants. It is not actually a virus, as far as we know, that infects animals at all. So, that particular virus at least has the structure. So, in thinking about reservoirs and where viruses come from, you may also want to think about viruses of other species and possible reservoirs in non-animal sources.

The other example of zoonoses that I want to talk about here are these viruses, particularly the virus Hendra and Nipah which are examples of the new paramyxoviruses which have occurred recently. So, let's look first at the situation with the Hanta virus in the United States. This virus was discovered in 1993. I don't personally believe that this gives us a lot of concern because it is confined to particular rodents, sigmondontine hosts. It occurs in these hosts, which mostly occur within the Americas. You don't find this Hanta virus, pulmonary syndrome, the serious disease, in the rest of the world, even though people have looked for it very carefully. There is a different disease caused mostly in Asia by the Hantaan virus. This particular virus, although it is serious and very deadly, is a dead end host within the humans. So, it is a virus which doesn't spread once it is in the human. As of this year, we have less than 300 cases in total that have been found.

We do know that there are a number of different viruses which are related, which occur in different rodents. The most common one is called Sin Nombre virus, or without name virus, which is all these red dots here. In addition, there are viruses in other parts, for example, one up here in New York which has a different rodent host. Instead of the *peromyscus maniculatus*, which these viruses have, this one on the east coast in New York has *peromyscus lucopus*.

There is a virus down here called Bayou virus down here in Louisiana, which has a host which is *erithamus pollustrus*, and Black Creek Canal virus, which occurs down in Florida, which the host is the cotton rat. So, you can see there are some different types here. What really concerned us was when we had some samples from Brazil. Two young boys had died in a family of three in an area near Sao Paulo. The virus turned out to be very similar to Sin Nombre. This is when we realized that South America also had problems. Down in South America, we now realize that there are numerous viruses of this sort which occur throughout South America. One particular problem here is that there has been some evidence with these viruses of person-to-person transmission, unlike the ones in North America. The evidence is so far fairly reasonable, but it needs further investigation to be sure that this is actually the case. So, these viruses essentially are spread throughout the whole of the Americas. All the viruses which are shown here in yellow cause very serious fatal disease, Hanta virus pulmonary syndrome.

This is Juquitiba, which was the virus where those two boys died in a family of three in Brazil. Lechiguanas, these are all viruses which have caused fatalities. Particularly Andes, down here, is a very serious virus which is recurring. These are viruses all of rodents, where the rodents spread to humans, but with the possible exception of Andes and these viruses down here, we don't think that they then spread further. Amongst the paramyxoviruses, there are quite a number of newly recognized viruses. This shows examples, and I have listed here the ones that affect humans. Hendra is known to have caused two human deaths. Nipah, more than 100, about 106 deaths have been recorded so far from Nipah virus, which is a virus of pigs which went into humans. They all originated here in fruit bats as you can see. Menangle I have spoken about already. Tioman is a virus which has been found on an island about 25 kilometers from Malaysia, where they are investigating Nipah virus. It is a virus of fruit bats which occurs particularly in a species called *Pteropus hypomelanus*.

Tupaia is a virus found in tree shrews, and these are all related, by sequence and molecular studies, to each other. Finally, we have Salem virus, which was found actually up in New York state, and is a new paramyxovirus which was isolated from peripheral blood mononuclear cells from horses but, when put back into horses, didn't cause disease. So, we are not too sure yet of the disease potential of this particular one. This is a whole family of viruses, but it includes some very serious examples. Nipah in particular, I think, is a virus of pigs which spread from fruit bats to pigs, and from pigs to humans. I already used the phrase in relation to the PERV, but this is obviously a very serious illness.

Just to go back a little bit in history to talk about how these viruses occurred, this man died of a very serious upper respiratory disease all caused by a virus called Hendra. He was the trainer in the Hendra Stables. His name is Vic Rao. He got infected from his horses. Fourteen of his horses died of the disease. We believe the horses got infected from fruit bats, but nobody knows how the virus moved into the horses. Certainly, he died and, shortly before that, another gentleman died who did an autopsy on a horse and actually developed a very serious encephalitis. He died in a place called Mackay. So, we had these two human deaths.

We know this virus, Hendra, can certainly kill. At the time the virus was first described, I was giving a talk in Lexington and there was a great concern, why was CDC over in Lexington talking about a horse virus. In fact, we thought this was not terribly relevant to the United States at that time, but I took the time to make sure that we got some samples of that virus from Australia and we made some antiserum. So, we had a collection of antiserum at CDC that we could use in case that virus was to reappear, which in fact, it did. Now, these are flying foxes or fruit bats, actually, in the air. You can see here what happened, the initial case of Hendra here, where the trainer died, is here, very close to Brisbane. There is Mackay, where the other gentleman died, who did an autopsy on the horse. We have one other recorded case of this virus, which was in a horse that died in Cairns in January of 1999.

A disease then began to break out in Malaysia, especially in the peninsula of Malaysia here, which at the time was thought to be an encephalitis. There was a lot of belief, because the virus was also causing deaths in pigs and encephalitis in humans, that this was probably Japanese encephalitis. The Malaysians began to kill lots of pigs because they thought they had a serious disease that was killing humans. That was certainly the right thing to do, but they also thought it was spread by mosquitoes. In actual fact, it turned out not to be a virus of mosquitoes at all, but another of these paramyxoviruses coming from fruit bats. To give you an idea of the economic importance of this virus, because it was killing so many pig farms, we have to go to the Wall Street Journal to get an account of what actually happened at CDC in terms of the time line of this virus. You can see that, from the first case, once we were called in toward the end of March of that year, we realized that what we had actually was a paramyxovirus. Then, taking that antiserum that we got from the Hendra virus, putting it onto those cells, we immediately got fluorescence and we knew that we had the same virus, or a closely related virus, to Hendra, which killed the horse trainer in Australia.

So, these were closely related viruses. They have a very, very long nuclear protein, very much like Ebola. It is a typical paramyxovirus, but it is much longer than the normal paramyxovirus. This is an example of the virus itself. We named it Nipah virus, after the village that the human cases from which it was isolated came. It occurs in peninsula Malaysia here. It has occurred over here, but principally in this area. We think it is a new genus of the paramyxovirus, which is capable of infecting pigs and also a wide range of other species. In humans, the main disease that was killing people was a fatal encephalitis and the respiratory symptoms were uncommon.

If you remember, the Hendra virus caused one death from encephalitis and one from respiratory symptoms. What we saw in Nipah virus was, in pigs, extensive respiratory symptoms, but in humans, principally encephalitis. You see necrotic areas in the brain of somebody who died of Nipah virus and then Zaki and his group, using immunohistochemical staining, initially we used the antiserum that we got from the Hendra virus, and we began to see this in the brains of people dying of Nipah virus in Malaysia. This is Nasset red stain, which stains up the presence of virus antigen here in these neurons. So, it was extremely valuable to have that. Here is an example of the endotheliosis that we see also in humans. There is a thrombosis and we can see evidence here of underlying blood vessels. So, these viruses seem to have a predilection for endothelial lining cells particularly.

In pigs, we see much more respiratory disease. They have an unproductive cough that the Malaysians spoke about hearing a mile away. They call it the mile long cough, because it was so loud from these pigs. Some of them have some lethargy and aggressive behavior, indicating some involvement of the brain, but principally this was a problem. You can actually see antigen here in this lung section. So, the evidence would suggest that simply with this explosive coughing, virus could be spread into the environment. We know that pigs that were imported into Singapore caused 11 cases of the disease and one death, and the spread appears to be principally from the respiratory system of the pig. This virus, as I said, can also kill dogs and it can also kill cats. Here is a dog autopsy going on here in Malaysia. The way the disease was dealt with really was to cull the pigs and get rid of all the pigs, in the same way we got rid of the chickens in Hong Kong. The Malaysian government decided that they should cull the pigs on infected farms. So, farms where they had cases of Nipah, all the pigs were killed, essentially, were shot. This picture shows the pig cull here in green, over top of the epidemiologic curve of the human cases. You can see that once more than a million pigs were slaughtered, there were no further human cases at all.

Here is an example of a virus that is spreading, probably one of the most dramatic examples of a lethal infection which can spread from pig to human. We need to know a lot more about it. We know now a

lot about the molecular biology. We know how to detect it. We have good methods for doing that. I think surveillance for such viruses is an important part, I think, for considering the use of animals for transplantation.

The link to flying foxes really came later, by collecting urine from flying foxes, and in that urine they found the virus. We don't really know what the initial event was, but somehow the pigs became infected with the virus from flying foxes. Then Nipah virus was then spread principally by the movement of pigs into different areas of Malaysia, where these began to occur. The particular flying fox we believe is important is this particular one, which is *Pteropus hypomelanus*, a black flying fox. This shows roughly the size of it. This is an EIS officer who is holding this animal.

In terms of the comparison of these virus genomes, here is a virus like measles, here is a parainfluenza virus. You can see that this paranipah group have a longer genome, considerably more intergenic regions, and they have a lot of information in there, more than 19 kb of information, which suggests that there are a distinct group. From a virological point of view, we now think of these as being a new genus and, in fact, the name Henipah genus has been suggested for them, to follow the Hendra and the Nipah. It has not been formally adopted yet.

Just to compare these finally, just to show you an example of how these are compared, by taking the nuclear protein gene, sequencing that, and then looking at how these changes fit together with other viruses. So, we are talking about Hendra and Nipah which are down here. They are quite isolated, apart from this tree shrew virus, *Tupaia*, from this group here, which is the measles-like virus. Then up here we have got the rubeolar group, which includes mumps, SV5 virus, mapuera which was isolated from a flying fox down in Brazil, a fruit bat in Brazil, Newcastle disease virus, here is Menangle and Tioman in this particular group here, and then the parainfluenza viruses in humans up here.

Now, what can we learn from this in terms of conclusions? There are a lot of observations and interactions, but I think we have to think, I think, first of all in terms of tropism and virulence. The first thing that I would like to say is that I think there is no doubt you can alter tropism and, therefore, virulence. You can move virus from one species to another by one or two single mutations. The best example of that is the canine parvovirus, but is also mutations affecting the cleavage site of influenza and also dramatically alter the host range, simply by altering the rate at which the human route is cleaved.

The second part is that you can also get re-assortment if a virus has a number of segments, particularly with influenza. We know the influenza viruses can re-assort from birds and infect pigs. We know that viruses of humans are already derived, ultimately, from viruses that infect birds. There are far more types affecting birds, and especially ducks, than any other species. Rarely, they can infect horses, too, but for the most part, it is pigs and humans that are susceptible to influenza.

Now, we haven't discussed this yet, but I am sure when Clive Patience and Dave Onions get here, they will talk about retroviruses. There is no doubt that host cell gene transduction, the ability of that virus to take a cell gene and move it into another cell, can affect pathogenesis. That is certainly how the oncogenes got moved around. We also know that a virus which is apathogenic in one species can be highly pathogenic in another. There are lots of examples, the herpes viruses in, say, moving from owl monkeys to squirrel monkeys where a virus can be lethal in another.

Then the statement is still true, I think, that for many zoonotic infections, humans are a dead end host. These dramatic examples that I gave to you have been extensively studied, and apart from that little evidence we have down from Andes virus down in Chile, there is really no evidence for extensive human

infection, or spread in the human populations, as that that actually occurs with HIV. In Nipah virus, in particular, we examined very carefully lots and lots of people who had very close contact with pigs, who had contact with humans who were sick, and none of these developed any infection. So, humans, generally speaking, are dead end hosts in the examples that I have described.

Finally, some implications for xenotransplantation. I don't think we understand virus tropism very well. I think we need a lot more studies to try to understand exactly what is resulting in these movements. As I said before, small changes can seemingly alter host range. So, it doesn't take very much to alter the host range of some viruses. Most cross species infections do not spread in humans. Finally, I am convinced that new viruses will continue to emerge through evolution and adaptation. What that really means is that we have to have very good surveillance systems. We have to be on the lookout. We have to maintain the appropriate reagents, such as that Hendra virus antiserum, in order to attack something once it appears.

We now have molecular techniques for looking for these things, too. Many of the parainfluenza viruses I have spoken about were actually detected primarily by a molecular technique in which you could look at the total messenger RNA present in a cell using a structure method that allows you to then find the viruses which are present. So, we are in a much stronger position to detect viruses in terms of molecular methods and other methods, but we need to be aware that it doesn't take very much for infection to spread from one host to another. Thank you very much. If there are any questions, I would be happy to answer them.

[Applause.]

DR. KASLOW: Thank you very much, both Drs. Allan and Mahy for the very informative and sobering overviews. We do have some time for questions. Let's open it up now to, first, the members of the committee, if there are any questions they may have for any of the speakers.

Agenda Item: Discussion.

DR. SYKES: I have a question for Jon Allan. You alluded to the fact that an alpha gal knockout animal -- pig -- would make retroviruses occur, if that lacked the gal epitope, and that this might increase the virus' ability to infect a human. Is there actual evidence that natural anti-gal antibodies have neutralizing activity against PERV, and could you summarize the evidence?

DR. ALLAN: It is too bad Clive isn't here. My understanding is that the antibodies to alpha gal are highly neutralizing, and that the primary reason that PERVs don't get into human cells in general is because of those antibodies. If you remove the antibodies, you may allow for the virus to infect human cells. So, if you deplete humans of this alpha gal antibodies, you may allow for the infection to occur. I don't know how good the evidence is. I wish Clive was here to comment on that.

DR. SALOMON: They did a series of studies, Robyn and Clive, where they took the PERV, they passed it into human cells, they demonstrated that they then could avoid the effect of the neutralizing antibodies if they passed them back in and pseudotyped them. So, they did all the standard virologic studies, demonstrating very clearly that these were neutralizing antibodies for PERV.

DR. VANDERPOOL: I have a question for both speakers. Your presentations remind us all that we live in a dangerous world where viruses are inventive, whether animals or humans are immunosuppressed or not. My question is, do the degrees of these infectivity possibilities, pathogenic possibilities, increase

with xenotransplantation and, if so, why. I mean, does immunosuppression make humans more susceptible to these genetic mutations of deadly viruses or not, and to what degree does the development of infection-free colonies of pigs protect us from many, perhaps most, of these naturally occurring viral outbreaks?

DR. MAHY: I think the best example we have other than transplantation is, of course, HIV/AIDS. There are quite a large number of viruses that could be listed that would not have been detected without the person being immunosuppressed. A simple example you may not have heard of is a virus called picoverna virus. It is a very small double stranded RNA virus. We looked in populations for this virus because we knew it existed in other species. It was only when we got a population of AIDS patients that we looked at the stools and there was this virus in large numbers of samples. There are lots of other examples, but I think the issue of immunosuppression is a serious one, because there is a constant surveillance going on within the human body to look for anything that is foreign. In many, many cases, we know that viruses are continually replicating and changing. We heard from Jon Allen about the high rates of mutation, especially in RNA viruses. Most of these probably never make it into an infection because they get cleared by the immune system. When that is not there, I think it is a problem.

DR. ALLAN: I would just add, the situation is that in xenotransplantation, it is a different route of transmission than one would normally have. So, that changes the whole situation. We are giving viruses that don't normally have an opportunity to find a human, and you are giving them the best possible route. Immunosuppression, while it is important, may be over-rated.

DR. MICHAELS: I might disagree a little bit on that. I think that you are possibly right, that in terms of the risk of transmission, perhaps the immunosuppressed environment isn't going to be it. I certainly think that in terms of disease manifestation, that immunosuppression is going to play a role, at least for the individual, if not for transmission.

DR. KASLOW: In fact, paradoxically, it may be easier to detect the occurrence of the virus under the conditions of immunosuppression than it might be otherwise. While the transmission may be no more or less difficult, it may be an early warning system, in which we could detect it more readily.

DR. SCHECKLER: I take from our background presentations -- very sophisticated presentations -- that really the focus is on viruses, in that parasites, fungi, bacteria, are not of great concern in terms of spread to the recipients, much less spread to the community. The other lesson that I think I am hearing from the two speakers this morning is that frequently the human is a dead end host here, and the risk for spread to other family members or the community is very small to non-existent with most of these viruses, if not all of these viruses. I think that is very relevant to the kinds of control mechanisms and so forth that have been suggested. Is that a valid statement? Is it the viruses that are the only soldiers to worry about here?

DR. ALLAN: That is partially my bias as well, because I am a virologist. I mean, the viruses are different because they require infection of a host cell. That is where a lot of the difference is, is just that the viruses have to cross species in order to replicate, whereas bacteria, parasites, many times don't need the cell. They just need the right environment. Obviously, bacteria and parasites are an issue in the transplant setting, and I think that Michael may address part of that issue. You can obviously treat with antibiotics. There are different treatments for bacteria and parasites, where there aren't those types of treatments generally available for viruses. The second point is that, while you can sort of eliminate a lot of the viruses in terms of being able to replicate that there is a dead end host, I wouldn't say that there wasn't any risk in the transplant setting. Some of these viruses, like retroviruses, it is very difficult to determine whether or not they are going to become pathogenic or not. If we do end up transmitting a

retrovirus or something related to that, there is the possibility that it could then get into the population. We don't know what that risk is.

DR. KASLOW: I guess I would comment that it probably would be worth our having a bacteriologist and a parasitologist and some other folks who might have a different perspective address us at some later point. Of course, bacteria and parasites are probably more readily detectable, at least visually, and I think that with the techniques that we have available now, we are probably quite a bit more likely to be able to detect them than we are viruses under the current circumstances. For that reason alone, one might be a little less suspicious of them, but I think we probably should, as a committee, hear from those who can tell us otherwise, in the future.

DR. MAHY: Certainly, my experience is entirely with viruses. As said by Jon, the control measures that are available in terms of bacterial infection, are quite different. Really, we have to be quite specific in the case of a virus infection to develop some antiviral drug or antibody to get rid of it.

MR. BERGER: I have one comment and one question. I don't quite understand this dead end host when we live in a world that is running rampant with HIV. I live in the San Francisco Bay area where the incidence of HIV is increasing. So, a dead end host doesn't seem to make sense in today's world. The second part, the question for Jon, we seem to deal with the transmission of an infectious virus at the front end when transplant actually takes place. My question would be, if there is a successful xenotransplant, are other risks down the road, the longer that that transplant or the host lives, in terms of something mutating or other viruses being introduced into that person just as we breathe and live?

DR. ALLAN: I think you are right. I mean, the longer the transplant is successful, in other words, if the organ is accepted, the longer the survival, you increase the survival and, if the virus is present, it may find itself another population. Now, you also have to consider that the more transplants you do, you are probably creating a greater risk. It only takes one virus getting into one patient to create a worldwide epidemic. The more patients who are transplanted over time increases the chances, at least in some viruses, that if they don't replicate very well, that over time, maybe one individual, for whatever reason, that virus may begin to replicate or adapt itself better than in another individual. You just increase the chances with the duration of the transplant and also with the numbers of people infected, I think.

DR. SHAPIRO: Jonathan, I am wondering if you could expand on one statement that you made that I found alarming. That is, if a retrovirus got into the germ line, that evolution could be affected. What is, in your mind, the significance of that risk?

DR. ALLAN: I just put it out there because I thought it was sort of a fascinating thought, rather than reality. I don't think that the chances are very good that that would happen. I just pointed it out because, really, if you look at – endogenous viruses are thought to begin with as exogenous viruses that find their way into the germ line. Once they find their way into the germ line, they integrate themselves at various sites. At various times – at least this is what people believe -- is that, over time, there are greater and greater numbers of integration sites. With the greater number of integration sites in a germ line, you could actually modify -- you could upregulate certain genes, you could downregulate certain genes. In the case of salivary amylase in humans, it is thought that the reason we have salivary amylase whereas other animals don't is because of the integration of an endogenous retrovirus near that particular gene, which actually activated the gene. I mean, these things are possible. I think they probably have very low probability.

DR. CHAPMAN: so, Jon or Dr. Mahy or whoever is appropriate, are you suggesting -- we have heard

here about mutations in a sort of scary fashion, a mutation of a gene and the virus becomes much more pathogenic or it becomes able to infect dogs instead of cats, or humans instead of pigs, and can be very deadly. If you, as a scientist, accept the theory of evolution, mutations are perceived to be the way by which species progress, and there are mutations that can be harmful, mutations that can be helpful. I am wondering, as I am listening to you talk, are you suggesting that these sorts of viral insertions, throughout history, may have been one of the factors leading to mutations in the evolution of various species, neither necessarily positive or negative, but resulting in change and variation and the potential for adaptation?

DR. ALLAN: I think it is just one of the ways retroviruses can alter evolution. I think that the theory of punctuated equilibrium is that it isn't so much a gradual change over a long period of time. It is major changes that occur within the environment or within an ecological niche that profoundly change evolution in a relatively short period of time, in an evolutionary sense. There is a study where they took chameleons or anole lizards and they took them and they put them on an island in the Caribbean where their environment was totally different. Within 10 years -- it was a very short period of time -- their leg size changed dramatically. So, it really has a lot to do with what is changing in the environment, whether it is in a human host or something else.

DR. VANDERPOOL: I think one of the good things about this meeting is that we are going to be talking about allotransplants as also fraught with infectious disease dangers. I think one of the questions that awaits us is, have these people who have been immunosuppressed, have they developed a series of diseases. Do we have any indications that there have been special mutations of viruses because of their immunosuppression. These talks indicate to me that allotransplants, even though we might tend to assume that they are "in house," that since they are human to human they are contained to human-type viruses, but the presentations we have show that there are these constant evolution and mutations of viruses across the globe, that affect humans who are not immunosuppressed. My question would be, to what degree would the insertion of pig organs into human beings be more or less dangerous than the insertion of human organs into human beings under immunosuppression.

DR. ALLAN: I think that is a question that has come up over the years. I remember Tom Starzl, at an Institute of Medicine meeting, raised that issue. He said, we have already got horrible viruses in humans. Why worry about a few more animal viruses. Spoken like a surgeon. From my perspective, what you don't want to do is introduce more infections that we don't know what might happen. You are increasing the disease potential by introducing new viruses and also you have got problems with diagnosis, prevention, control, all those things that occur that you have to deal with. As a virologist, the potential is there. Look at the AIDS epidemic that everybody keeps throwing out there. That virus wasn't around 30 years ago and now, as Brian has shown, it is 25 million people in Subsaharan Africa. That is something we want to avoid.

DR. MAHY: I think one of the important issues here is that we understand human infections and the nature of human infectious disease much more thoroughly than we do for some of the transplants such as pigs. There needs to be a good understanding of the pig. We know a lot about it, but a lot of this stuff has only come about since xenotransplantation was proposed. Many of the PERV studies and analyses are quite recent, and there are very wise and very good studies that have been done. I think as long as we understand what we are doing, that is the main thing. You are taking a system that would have very little basis in terms of knowing the real viral overview of what is going on in pigs and humans.

DR. VANDERPOOL: I would be scared to death if we were looking at transplanted fruit bat organs into human beings. I think Jonathan Allan has led the charge of worries over primate organs in human beings. The real challenge is, given the dangerous world of viruses out there, can we feel that we have a

reasonable degree of control over pig organs in the human being. Just very specifically, do we have a good grasp of the number of viruses and viral transmissions and mutations between pigs and humans and can we get good control over that. At one point, I wake up at night thinking, well, we are pressing toward assurance to the point of near protection in terms of no viral infections from pigs to humans. Yet, the world out there show that infections are multiplying all the time. That doesn't get us off the hook, but it says that some of our worries, if we can modify them as much as possible, we are still going to have some worries out there, because there is always this inventiveness of the viral organisms to mutate and cause infectivity and pathogenicity.

DR. SYKES: I found Jon's classification into acute and persistent infections very helpful as a way of thinking about this. As I understood Dr. Mahy's talk, all of the sort of acute infections that readily cross species that you described have all been acute in the host species from which humans have been infected as well. There has always been another animal transmitting the virus to the donor species. My question is, if you had a closed colony of pigs and really didn't have exposure to fruit bats and could keep out other species, as well as humanly possible, what is the chance of their being a virus within the colony that could cause this kind of an infection in a human? It seems to me that you would know about it if it produced illness in the pigs. The only way that it could elude attention is if it was completely non-pathogenic in pigs, in which case it would be a persistent virus, rather than one of these acute viruses. Do we really not have to worry about these.

DR. MAHY: I think obviously retroviruses provide the biggest concern. In a closed colony in which the retroviruses you had to deal with, there was no possibility of a porcine retrovirus establishing itself, you probably would go ahead. Your only concern then is that you would have pig cells obviously in a xenotransplant that might be susceptible to some other infection that the human may encounter later on. In terms of the initial transplant, I think it is possible to prepare a closed colony which would be at least free of things we know about. We can't do more than that.

DR. MICHAELS: I just wanted to comment in that regard. I think it is such an important part that Megan brings up, about having the closed herds, and I am sure Dr. Swindle is going to talk about it later. Without the xenotransplant conferences occurring, I am not sure that such attention to the husbandry and having really quite good attention to the closed herds would have been made. I think that is important as well.

DR. KASLOW: Sharon, I think we will take your comment and then perhaps we should move on.

DR. KIELY: Very briefly, I think Dan Salomon brought this up at our last meeting, the fact that this is going on, xenotransplantation in some form is going on across the world. He brought up the public health issues of that. Dr. Mahy brought up the issue of surveillance and the critical importance of surveillance. I think one of the critical issues we need to focus on as a group is what is going on in the world, what are the public health implications of that. We talk about closed colonies and protected herds and animal husbandry, and those issues are only a small part, when we deal with those xenotransplants and clinical trials that go on in this country. I think we need to focus in a global sense on the public health issues.

DR. KASLOW: Thank you very much. I think we will move on, then, to our next speaker, who will address the infectious agent transmission in the allotransplantation setting, infections after allotransplantation, lessons learned, Marian Michaels from Children's Hospital in Pittsburgh.

Agenda Item: Infectious Agent Transmission in the Allotransplantation Setting. Infections After Allotransplantation: Lessons Learned.

DR. MICHAELS: Thank you. As we entered the modern era of transplantation in the last decade or so, with the amount of immunosuppression used to prevent rejection, the intimate relationship with infections became widely apparent. While we do know a lot, and a great deal of progress has been made with infections, with transplantation, in fact, I think people sometimes give us more credit than we have, and infections still remain, today, a major cause of morbidity and mortality after allotransplantation.

What I would like to do in this 20 to 30 minutes is review the infectious risk with transplantation to sort of give a basis of where we are, and have a context for understanding infections that might occur with xenotransplantation. To do that, I will characterize the risk factors, as some of these will be the same with xeno. I will talk a little bit about some of the specific infections to highlight points made. I am really not going to dwell on diagnostic tools because of time constraints, and spend a little bit of time on the prevention and prophylaxis that we use today in allotransplantation. I just wanted to mention several very simple concepts that I think important while we go through this, the first being that infections are a risk with the use of any biologic tissue, whether it be from human or animal. If we don't know, or we don't know how to look, we aren't going to find it. Finally, this can never be a static process. We must have enough elasticity that we can modify our protocols as we go along and as we are faced with new findings and data.

Let's turn to some of the risk factors. For allotransplantation, the risk factors can be divided into those which are pre-existing, those that occur during the operative – we are really going to highlight on organ transplantation here -- those which are interoperative factors and those that occur after transplantation. The pre-existing factors are largely defined by the underlying illness. What type of organ is going to be transplanted? Is it a liver transplant due to hepatitis C end stage liver disease and the risk of infecting the new organ? Is it a cystic fibrosis patient who harbors multi-resistance to the monads that could then come down from the airway and infect the new lungs? The severity of illness of the person undergoing the transplant. Is this a patient lingering in the intensive care unit who, time and again, good studies have shown will have increased risk for bacterial and fungal infections early after the transplant procedure?

As a pediatrician, age is something that I always like to bring up. The way the host will respond to new infections is somewhat dependent on the age of that patient. In some ways, that really reflects the pre-existing immunity which that person brings with them at the time of transplantation. Have they been on earth long enough to have experienced or been exposed to infectious agents and developed protective antibodies and T cell immunity to them. Donor-related issues we will come to in a moment. The interoperative risk factors, the type of reconstructive surgery used, what types of technical difficulties have occurred, duration of surgery. A reflection really on difficulties that might have occurred with transplant also is a way of predicting the risk for infections early on afterwards. Likewise, the quantity of transfused blood, not only the risk of transmission of the infections from our transfusions but, likewise, a reflection of the difficulty and events that might have occurred during surgery itself.

After transplantation, immunosuppression, by far and away, is the biggest risk factor for disease from infections after transplantation. Technical problems -- was there a hepatic artery thrombosis, anastomotic problems after lung transplantation – all of these, too, incur an infectious risk, but are less so than the immunosuppression. The presence of in-dwelling canulas, breaking through that protective barrier of the skin, and nosocomial exposures are ones we too often have to deal with, unsuccessfully, I might add, at times. Now, the sources of microbial agents are multifold. They can be from the endogenous flora of the recipients, those pseudomonads in the cystic fibrosis patient. They can be from the environmental flora,

both from the hospital and, with that patient eventually leaving the hospital, from the community -- influenza outbreaks, RSV season, respiratory syncytial virus. Finally, they can come from the donor, either the organ or the blood products that we use.

Now, I know I am talking about allotransplant, but I just wanted to bring back into focus some of the early data on the xenotransplantation experiences. Both Keith Remsa and Thomas Starzl, in the 1960s, describing their experience with chimpanzee and baboon kidney xenotransplants, found five of the six fatalities to be largely contributed to from infections. Now, the type of infections that occurred were bacterial in nature and were acute after the transplant and, as such, were likely due to the environment or the endogenous flora. Likewise, Thomas Starzl's two baboon liver xenotransplants into humans died with overwhelming infections, the first with disseminated aspergillus, a hospital environmental pathogen that disseminated because of the immunosuppression used, and the second, with polymicrobial gram negative sepsis, organisms that came from the patient's own gut, because of technical difficulties with the procedure. These cases highlight the fact that endogenous and environmental sources of infectious agents can be there and can be the same, both after allo and xenotransplantation.

Let's turn to some specific infections and their sources. As we mentioned, the donor transmitted infections are ones that are of particular note for allo as well as for xenotransplantation. While we talk about these as if we have known about them forever, in fact, that really is not true. It was with good experience and a lot of experience from the surgeons themselves, from the transplant physicians, from the laboratory personnel, strong epidemiologic studies that finally made us recognize that latent and, perhaps more correctly, persistent infections that could often be asymptomatic that is the source of the organ, could reactivate in the new host and cause substantial disease.

The types of infections, as Jonathan had already pointed out, can be broken down into latent or persistent organisms and into acute infections. The latent and persistent ones, the herpes viruses, in particular cytomegalovirus, CMV, EBV -- Epstein-Barr virus -- hepatitis B, hepatitis C, parasites are important -- I will talk about those a little bit -- toxoplasmosis, malaria, others such as HIV, histoplasmosis, tuberculosis, can all be donor associated. The acute infections happen less frequently because, as people have pointed out already in discussions, we are often able to recognize those. Still, bacteremias and acute viremias that are not recognized in our cadaver transplants, have been transmitted with dire consequences to the new recipients.

Let me just spend a few minutes talking about a few of these organisms, so that we have a clear understanding. CMV infection, a rather benign infection in the normal host, can cause disease after transplantation, from primary reactivation of their own infection or re-infection with a new CMV strain. Primary infection has the greatest morbidity and mortality and is what we call the mismatch state being the highest risk. Mismatched, where the donor is seropositive for the virus and brings it over with the organ into a recipient who is naive, who has no previous contact with CMV, doesn't have antibody protection and, more important, does not have T cell immunity. In this setting, we almost always see a severe reaction if we do nothing. There is an increased risk of disease, again, with heavy immunosuppression, times of rejection treatment, and re-infection is often from the donor as well.

Here, just to sort of highlight the point, this was from the bronchial alveolar lavage of a youngster who was CMV negative prior to her transplant, received lungs from a CMV positive donor, and presented to us with high fevers, rapid respiratory rate, need for oxygen, diffuse findings on her chest X-ray and, in the bronchial alveolar lavage, this huge cytomegalic cell with the CMV inclusions in the cytoplasm. Now, despite knowing that CMV is in our patients, and despite knowing that every organ transplant that we do, that there is a risk between 10 percent to 45 percent of our patients having CMV disease -- small

bowel, lung, liver, heart, pancreas, kidney -- we do persist in transplanting, doing these mis-matched transplants, because we don't have choices to wait for other organs. We have been able to modify the disease by good treatment, by preemptive treatment, screening the patients, and I will talk about that a little bit later, but we still have it.

Epstein-Barr virus, another member of the herpes virus family, again, donor transmission recognized by epidemiologic evidence and more clearly proven by DNA fingerprinting, that the virus, the EPV, that caused PTLN, post-transplant lymphoproliferative disorder, in the patient, was the same EPV that was asymptomatic in the donor before the transplantation. This can present in multiple fashions. It can be mononucleosis just as we see out in the community, fever, malaise, pharyngitis, and you can see this really exuberant, exudative tonsillitis on this youngster, who responded to us just decreasing her immunosuppression. It can also present with disseminated fatal lymphomas and proliferation throughout the patient that we cannot get on top of.

I wanted to just mention the alpha herpes viruses. While they are important to know about for reactivation of the endogenous organism of the person receiving the transplant, in fact, herpes simplex and varicella zoster -- chicken pox -- are rarely donor transmitted. The reason for that is that they live in their chronic persistent state. Their latency is in the sensory ganglion, and are not in the tissue or the cells being transplanted. So, they are not as much a risk. So, no one thinks that only herpes viruses can be donor associated. There are a number of other viruses, some of which have already been brought up.

We have done a lot of good with our transplantations of organs, tissues, blood transfusions. Our history has too often been littered with having unwittingly transmitted the viruses, hepatitis B, HCV, hepatitis C, and human immunodeficiency virus. The vast majority of times they occurred prior to our identification of the agent, of the virus, and prior to being able to therefore institute good screening techniques. However, with that said, even in the days of having good screening, problems can occur. R.J. Simmonds and colleagues from the Center for Disease Control reported a single donor transmitting HIV to all four recipients of his organs, and three of the four recipients of his bone grafts, despite testing negative for HIV. Now, every test has a limitation. Accordingly, he must have been at a point where he did not yet have detectable antibodies, but we must recognize those limitations, or we won't be able to do things about it.

People have brought up the fact, are we just worried about viruses, so I wanted to bring up this youngster, which some of you have heard me talk about before. This was one of my first transplant patients when I came to Pittsburgh. This young girl presented to us three months after her heart transplant with inability to use the right side of her body and blindness reaching out for her teddy bear. She could no longer find it. When we did an MRI scan, we found these huge lesions in her brain. The biopsies of those areas and of the heart showed this little creature, which is toxoplasma gondii. I have the life cycle written here, but just briefly, toxoplasma's definitive host is the cat, but humans, as well as other animals, including monkeys and swine, can be infected by ingesting the oocyst, the reason pregnant women are not supposed to change the kitty litter. It can also be from the soils that have been contaminated with defecation from cats. Once inside a non-cat species, the oocyst goes into the trophozoite stage and it starts traveling around the lymphatics. In response to the animal immune system, as well as being unable to find the ability to sexually replicate outside of the cat species, it goes into muscle tissue and into any organ, actually, and forms a cyst, where it remains for the rest of that individual's life. It can go anywhere, but it likes to go to a couple places, the heart being the number one place of tropism for this protozoa. So, taking a heart from a person who had toxoplasmosis years ago, putting it into an individual who had never seen this organism, had no immunity, then immunosuppressing them, allows this latent organism to come out of latency, reactivate and go throughout the body, causing the dire deeds that we

saw.

Now, what this taught me, and another case that came shortly on the tail of another toxoplasmosis in another of our patients is that we were not screening. Not only were we not screening, but most centers in the United States were not screening for this. There was treatment that we could use prophylactically. With that in mind, we modified our protocol based on these new findings and subsequently, any mismatched states that we have, we have been able to institute prophylaxis and have avoided this fatal disease.

As I mentioned, I just want to touch on diagnostic tools to try and get us back onto a good time frame, but really, I have said it before. We must recognize that each specific test has attributes. It tells us something. It will also have limitations, and we must learn from those experiences and recognize those limitations, or we will regret them. I think I left in one example. This is CMV. That is the virus I work with, so I use it a lot. This is CMV growing on a fibroblast, and it is a great way to show you that there is replication going on in whatever tissue you ended up submitting for viral culture. This takes 10 to 21 days. In the old days, if we had to wait for that with transplantation, our patient either got over the disease themselves, or was already dead. It clearly was not adequate.

Newer techniques using antigen stains for shleavial shown on this, for antigenemia studies in the peripheral blood, polymerase chain reactions, have all shortened that time down to 24 to 36 hours to get an answer about CMV. Likewise, for EBV, we really used to have to rely on antibody development in the patient. The immunosuppressed patient did not always develop an antibody response, even when infection occurred and, most often, when they did develop the antibody response, they already had the disease. So, moving to PCR techniques has helped us tremendously.

With that, let me just spend the last few minutes on prevention and prophylaxis. The way we approach infections in our transplant patients, because we know that they will have infections, is to do a pre-transplant evaluation. We need to do a physical and history on the patients, plant a PPD to look for evidence of tuberculosis, which would delay their transplant but allow us to treat them appropriately beforehand, serologic screening, to know what viruses might reactivate in their body, but also what immunity do they have so we don't have to worry so much about those organisms. Then, I left it off, but most importantly, in this period, we have the chance to do education and counseling, to tell them about the risk of infections, so they don't think that transplantation is a pure cure of the disease that they are suffering from, and that we do have to watch.

Since I have been involved with xenotransplantation, I also counsel them about the potential of transmitting these diseases to their close contacts and loved ones. Also, we take advantage of the time awaiting transplantation to update their immunizations to give them protection. The donor screening is obviously quite critical, and has changed over time to become more extensive. It is somewhat dependent on what organ, what tissue, or if it is blood that is being transplanted and, in general, will include HIV, HTLV, CMV, a test for syphilis, hepatitis C, hepatitis B, and then in specific situations we also look for other viruses of concern – Epstein-Barr virus, toxoplasma *gandii*.

Blood cultures and urine cultures are taken on every organ donor, and patients who are going to be donors for lung transplantation, we also take a sample from the airway itself, to know what they bring over.

What has changed over time -- and if anyone has been giving blood for years and years now -- they will notice that the list of questions on life style and experiences has increased substantially over time --

travel, drug use, ethanol use, whether there have been recent tattoos or skin piercing, previous transfusions or previous receipt of organs or human growth hormone, are all questions now that are asked of donors. What, then, becomes acceptable in the donor, because they will come over with infectious agents. It depends on what substance is being donated. What may not be acceptable for a kidney candidate who is stable on dialysis may be very acceptable for the patient who is comatose from liver failure or waiting for a lung transplant, where few organs are available. Certainly many things that would never be allowed for blood or tissue transplantation in the donor will be acceptable for these patients who do not have enough organs available. Finally, some of the acceptability can be donor/recipient specific. While I will use the CMV positive donor for almost all of the organs, even if the candidate is not CMV positive previously, we will not do that for a small bowel, because the disease that occurs is too high. We could use a CMV positive donor of small bowel in an already-CMV positive recipient. The same with hepatitis C. You could use a hepatitis C positive donor in patients that are already hepatitis C, although that, too, is somewhat controversial.

Finally, just to end, prevention is certainly key. We use perioperative antibiotics. Almost every center does some form of prophylaxis with trimethaphin sulfamethoxazol against pneumocystis carinii from the environment, prophylaxis with ganciclovir or a consistent monitoring system for CMV, monitoring for EBV, monitoring for toxoplasmosis. All of these systems are defined by what we know is coming in that particular group of patients.

Just to conclude, we need to screen the donors and the recipients, determine what our contra-indications will be, and for which situation those apply, prophylax wherever possible, perform serial evaluations to see if we were successful, treat when infection is found, and re-evaluate our protocols as time goes on. Let me stop there, and I am happy to entertain questions.

[Applause.]

DR. KASLOW: Thanks, Marian, for that very succinct presentation. Let's take a question or two just about her presentation, clarifications.

DR. ALLAN: In a xenotransplant setting, would you select patients based on the viruses that are present in a recipient versus, let's say, in a pig? If you know that PERV is present in a pig, would you exclude human recipients who have HTLV or have a retrovirus, because you are concerned about recombination events or things like that?

DR. MICHAELS: That is an excellent question. I had been thinking about it more from the standpoint that the viruses that are specific to an organ donor for a xenotransplant scenario, that the human was always going to be naive. I hadn't really truly thought about it in terms of if they had an exogenous retrovirus. I think especially early on in xenotransplantation – I have to think about that. I was going to say that, early on in xenotransplantation, I would suspect that we should avoid some of the retroviruses. In fact, I certainly was intimately involved in specifically transplanting a young man who had HIV, and in fact, the transplant was specifically an attempt to give him an immune system that would be resistant to that retrovirus. I think it is case by case. I am not sure that we can say the endogenous retrovirus would necessarily combine more frequently with the exogenous retrovirus of the human.

DR. KASLOW: The chairman has the prerogative for the last question.

DR. VANDERPOOL: First, to congratulate each of these speakers, Drs. Allan, Mahy and Michaels, on excellent presentations that are fraught with a great deal of provocative insight. Marian, I don't want to

put you on the spot, because the whole committee is on the spot on this one, but given the compromised health status of those who receive allotransplant, do you think that recipients of xenotransplants would be ipso facto less compromised in terms of their health, that this would be a sort of even set of infectious disease worries, or do you think that it is possible for those receiving xenotransplants to actually be less susceptible to infection due to their receiving organs at an earlier date.

DR. MICHAELS: If I understand the question correctly, because we would be able to transplant individuals before they became so severe, that we would have less infectious risk, I think that would be one of the benefits that would occur. Not only that, the ability to screen the xenotransplant source animal and prevent a number of the infections from being in them in the first place would be a benefit. Having said that, I think there will still be organisms which we are unaware of and will come over. It becomes a balancing act and I don't know exactly where the scales are tipped. I certainly think that those are some of the advantages that xenotransplantation would give.

DR. KASLOW: Good, thank you. We are going to take a brief break until 11:30. Then we will resume, not with the schedule that you have, but we will pick up with the xenotransplantation source animals and products at that point. So, return at 11:30, please.

[Brief recess.]

DR. KASLOW: We are going to continue now with the next section. We will have time for probably two talks before we break again for lunch. We will start now with xenotransplantation source animals and products out of the original order with the first talk from Eda Bloom on FDA requirements and recommendations for xenotransplantation animal sources and products.

Agenda Item: Xenotransplantation Source Animals and Products: Minimizing the Risk of Transmission of Infectious Agents. FDA Requirements and Recommendations for Xenotransplantation Animal Sources and Plants.

DR. BLOOM: I am going to try to go as quickly but as carefully as possible through our current requirements and recommendations in xenotransplantation. I will first give you a brief introduction and then I will talk about the varying levels at which we think it is important to control the pre-xenotransplantation procedures, so that infectious disease can be minimized.

I put this up again, even though I showed it to you all last time, because I want to make everyone comfortable with our current definition of xenotransplantation. That is: any procedure that involves the transplantation, implantation or infusion into a human recipient of either live cells, tissues or organs from a non-human animal source, or human body fluids, cells or tissues or organs that have had *ex vivo* contact with live, non-human animal cells, tissues or organs. At least a couple of these, particularly in B, tend to get forgotten when we speak about xenotransplantation. The xenotransplantation products are the live cells, tissues or organs that are used, whether they be human that have been exposed *ex vivo* to non-human, or whether they be non-human from the start.

Examples of xenotransplantation include organs which we like to talk about but don't have currently anything under IND for that. Implantation of non-human cells or tissues, some of that you have heard about last time. Also, the idea of extracorporeal perfusion of human blood through non-human cells or organs that may be contained in a device or not. The administration to human recipients of human cells that have previously been cultured *ex vivo* in the incubator with non-human cells, and those human cells that may contain contaminated cells or not are then re-transplanted back into a human recipient.

The way we regulate xenotransplantation, we take advantage of all the tools we have at hand, including the use of the current regulation, all of which can be found in Title 21 of the Code of Federal Regulations. In addition to those regulations which are the requirements, we have a lot of recommendations. The recommendations include those made in the PHS guideline which you heard about last time. Then, FDA has published three guidance documents up until now. One of them deals specifically with non-human primates. We discussed that last time. One is dealing with blood donor deferral and that one is currently under revision. One which I will talk more about today -- that is, the guidance for industry -- is on source animal product, preclinical and clinical issues, concerning the use of xenotransplantation products in humans. The clinical issues you will hear about tomorrow, because those come into play after the procedure. In addition, FDA uses appropriate other guidances which are relevant to xenotransplantation, and the International Conference on Harmonization guidance documents, or ICH documents.

First, the preclinical considerations. In general, the principles that apply to any preclinical development of any product preclinically would apply to xenotransplantation. That is, to identify what toxicities may occur, to show safety in an efficacy model, and so forth. A primary intent in particular in xenotransplantation, preclinical studies now, is to identify potential clinical risk factors that may influence infectious disease risks. We recommend that the similarities between the animal testing strategies -- that is, the preclinical -- and the human testing strategies -- that is the clinical testing -- be as similar as possible, and that the similarities include those at the level of the test substance, the route of administration, the dosing, that clinically relevant immunosuppression be used in the animal model, and that a test to evaluate the cell, tissue or organ type being examined for us in humans be developed in animals. In particular, as far as infectious agents are concerned, and as you have heard, there are infectious agents that are pathogenic, that are non-pathogenic or latent.

Any of these may be a potential cause of concern for disease in an immunosuppressed patient, or perhaps even in a non-immunosuppressed patient. We need to consider the microbiologic burden of xenotransplantation products, and the immunosuppressed state of the recipient. We would ask that there be careful monitoring of the animals, and we are talking again about preclinical studies, so in this case the recipients are animals as well, and we need to take note of any early signs of infection. We ask that people doing preclinical studies in preparation for clinical trials consider developing procedures that they can use then to assign a cause of mortality or morbidity in their preclinical model.

We will now move on to the characterization and considerations involved in the source animal selection and use. This will be a slightly longer segment. There are two major considerations in the selection of the source animal. The potential for transmission of infectious agents to humans, which is really what today's meeting is about, as well as physiologic compatibility, which we won't really discuss today.

We ask that endangered or protected species be absolutely excluded from use as xenotransplantation source animals. We also have, as I have alluded to, a document on non-human primates which, at the current time, suggests that there is insufficient safety information available for FDA to assess the risks of the use of non-human primates and, therefore, protocols proposing the use of non-human primates would be put on hold. We also ask that animal welfare concerns regarding experimental use of animals be applied. For example, the animal welfare act, which is a law, the PHS, if PHS funds are received, is also a law. We ask that the standards provided by AAALAC, the American Association for Laboratory -- I am sorry, I don't remember the acronym, but I am sure that other people here do -- be applied, so that the care of the animals will be as fastidious as possible. As far as source animal origin, we want to be sure that the animals being used have a documented history.

Now, in some cases, we see protocols where cell lines are used, and I think we have talked about 3T3 cells as being used in xenotransplantation. That cell line has been around for a long time, and we are unlikely to get information about a particular mouse that donated that line, but we do ask the best characterization as possible of the cell line itself.

The animal and herd qualification is something that we are very concerned about and we ask that closed herds with documented health screening programs only be used. We prefer that animals originating from outside the United States or their first generation offspring not be used as source animals, unless a sponsor can supply a really compelling reason why, and that is unlikely to happen. It is always possible to establish a colony in this country. Free ranging and wild caught animals are basically out of the question. There are two major concepts now that we like to consider when we are talking about animal health and animal husbandry. One is the facility, which needs to be adequately designed. The other is that there needs to be a program to minimize the exposure to infectious agents. As far as the facility, we recommend barrier facilities that would provide an environment where you could have animals free of designated pathogens. Such a facility should be built in accordance with AAALAC and the National Research Council guide for the care and use of laboratory animals. In addition, the facility is subject to regulations by the FDA under 21 CFR Part 600, subpart B, establishment standards which include certain requirements regarding animal care and personnel caring for those animals.

The program of operation to minimize exposure to infectious agents is crucial. It needs to be in accordance with documented standard operating procedures or SOPs. The health screening is another crucial component and we suggest that sponsors have experts that can devise a list for screening, including infectious agents that are known to be present in the source animal, and reasons for agents being excluded from screening if they are excluded. Individuals to compile such a list should include infectious disease experts, virologists, microbiologists, accredited microbiology laboratories and vets. We ask that the animal husbandry be conducted using a sentinel animal program so that constant monitoring is possible. Finally -- I think I am going to mention this again shortly -- is that animals that will actually be used as source animals for xenotransplantation should be quarantined for three weeks prior to their use. That is quarantine over and above the barrier in which they are housed.

The program of operation has other aspects as well. As far as the health care of the animals, records need to be kept documenting any illnesses, vaccinations, and only sterile techniques should be used for manipulation of animals being raised as potential sources for xenotransplantation. Sources of feed need to be the subject of good record keeping and rendered or recycled mammalian materials should not be used to feed source animals. Also, of course, feed with significant drug contamination or herbicide residue should not be used. Care takers need to be monitored for their health and they need to operate according to SOPs, as well as be properly trained. We also ask that traffic be controlled. That is not so different from most experimental animal facilities.

The particular source animal qualification is now the next level that testing will happen at. Animals need to be tested for the same infectious agents that the herd was tested, plus additional testing, as we will talk about shortly, as recommended for final product. We recommend that if possible -- because we realize this may not always be feasible -- that the biopsy of the xenotransplantation product, or other relevant surrogate tissue, be obtained for testing. That tests be performed as close as possible to the time of harvest and not more than three months before harvest of the xenotransplantation product. If more than three months have elapsed between the testing and the use of the product, then we ask for re-testing. In devising what test will be used, one needs to consider the nature, the timing and the results of surveillance of the herd in designing the actual source animal qualification program, and again, the three weeks.

As far as harvesting the xenotransplantation product, the sponsor should use a documented process for procuring and screening. Documented means it needs to be written out as an SOP or as some other formal protocol. They also need to keep good records. They need to document the results of harvests that have happened -- let me start over -- documenting results supporting successful harvest of xenotransplantation products that meet lot release criteria. So, they need to have shown that they can obtain xenotransplantation products that are appropriate to be used clinically, and I said according to SOPs already.

If it is necessary to transport animals, which occasionally it may be, if the animals are raised at a site distant to where the harvest is going to take place, we ask that such transportation include dedicated vehicles, and that those vehicles have barriers that are at least as good, preferably better, than the barriers that are maintained by the facility, because of the risk of introducing new and fun infectious agents in transit.

As far as the animal surveillance, it doesn't stop when an animal is used for xenotransplantation. We ask that a biological specimen archive be maintained for 50 years, and I will talk in a second about the 50 years. The archive needs to include samples of the product, necropsy samples from the animal if there has been a sacrifice, and blood. According to the PHS guideline, we ask that sponsors maintain three sets of samples or archive three sets of samples. One set would be dedicated for use by the Public Health Service if needed. One set would be used for recipient health care purposes, and the third could be used by the sponsor as they deem appropriate. Again, health care records need to be maintained for 50 years. We have often been asked where this 50 years comes from. You have heard about a bunch of other viruses this morning, one of which is HTLV, which is thought to have a latent period of 35 years, plus or minus. The 50 years was an initial attempt to try to capture the longest possible latent period that we think would be reasonable without saying just monitor and maintain records forever. The 50 years could be changed at any time, if evidence arises to support such a change.

Now, as far as the products are concerned, let's move on to that. I am going to talk briefly about the kind of characterization that FDA likes to see of products. This will include more than infectious disease, just because I think we need to begin to think in terms beyond infectious disease. Products need to be tested for safety, for identity, meaning that you got what you think you got, for purity, to say you didn't get a lot of what you don't want, and for potency, to say what you got works. There are assays that are recommended for licensed products in the Code of Federal Regulations. It is not necessary to use exactly these assays in product development, but it makes it easier if relevant assays exist that are in the CFR. Two general classes of xenotransplantation products exist for the purpose of testing. There are those that are used immediately after harvest, such as would happen with an organ, and there are those that can be stored and processed, such as cell lines that may be established, or cells that may be frozen.

For products used immediately after procurement, it is clear that the source animal considerations are even more essential than those that can be stored or processed, because that may be all the testing you have before you actually do your xenotransplantation procedure, since the tests on the final product will not be available prior to use. In such a case, a biopsy of the product or relevant surrogate is recommended. Now, the kinds of safety testing that we ask are basically sterility testing, including fungal and bacterial sterility. So, yes, we are concerned with organisms beyond viruses, as you have heard some good reasons for this morning, as well as mycoplasma in viruses. We ask that products be tested for endotoxin or pyrogen. Pyrogen is a substance that causes fever. Often pyrogens are bacterial components that have been left behind when bacteria are no longer present. A large class of these are endotoxins, which are a component of bacteria. So, we test for that because they can cause adverse events, even in the absence of bacteria. Then again, for identity of fresh organ transplants, perhaps

histology -- this is just a suggestion -- could be used for whole organs. Even though the xenotransplantation product may already be in the patient or have been used, we still ask that assays continue for the full culture period or for whatever is necessary to obtain results, and that results be recorded.

For stored or processed xenotransplantation products, the results of tests for safety, identity, purity and potency should be obtained if feasible, before the product is actually used. Safety, again, is bacterial and fungal sterility, mycoplasma and virus tests. Identity can be such tests as cell markers, species verification. Purity would also include an assessment of contaminating cells. So, possibly if you have markers available, you need to show what cells may be in your product that you may not want there. A potency assay could be used to detect biological activity such as insulin production or whatever, and the development of a potency assay may proceed as the product development continues.

I am going to talk a little more in depth about the microbiological testing, since that is really the subject this morning. As a general concept, we ask that sponsors adopt up-to-date assays. They need to support their assays, however, with data. Those data need to show the sensitivity, specificity and reproducibility of such assays. Again, another general concept is the 50 year archive, in this case, of the final product. For xenotransplantation product used immediately, tests should still be completed, as I said, even if the results won't be available, for stored or processed xenotransplantation products.

We may ask, depending on the amount of time the processing takes, for frequent and multiple tests at different levels. For example, if cultured cells are used, we might ask that microbiological tests be performed with the initiation of the culture, before cryopreservation if there is a prolonged culture, as well as, as late as possible during a culture period, such that final results or at least useful information will be available prior to the release of the product. In the case of cellular therapies in humans, and likely as well when animals are used as sources, we ask for testing no more than two or three days prior to clinical use, and then an interim sterility test can be used as some means to gauge the sterility of a final product. We also ask that, however many tests you have done, that tests also be done on the final product.

Now, I alluded to the fact that some xenotransplantation products are combinations with devices. In some cases, physical barriers are claimed to inhibit the transmission of infectious agents. However, such claims need to be validated, and the validation should include conditions of normal physiologic use, as well as stress under biologic or physiologically stressful conditions. For example, if a pancreatic islet is encapsulated in an allogeneic or other capsule, we would ask for validation to show that pressure on the capsule, extreme temperatures and so forth, would not violate the barrier. Microorganisms representative of infectious agents potentially present in a xenotransplantation product should also be used, and they should consider the size and plasticity of such agents. Finally, other agents other than microbiological agents should be used to demonstrate the integrity of any device that is used as a barrier, including permeability or using various particles. It could include viruses, but other particles with differing properties such as shape, hydrophobicity, charge and size.

As far as the assays are concerned, in general, the choice of test, we realize, would vary depending on the animal source, and that would include the species, strain and geographic origin, because some diseases may be endemic in some areas and absent in others, as well as consideration of the histological types of tissue, the processing of the tissue prior to use, and the proposed use for clinical indication. Tests should be included that cover the agents that are present in the source animal and, when possible, we encourage anyone developing a clinical protocol, to discuss their test with CBER.

As far as bacterial, fungal and mycoplasma tests, again, those are in the CFRs, but alternatives may be used with supporting data. We ask that gram stain be performed on the final product, recognizing that this is an insensitive test, but has been shown to detect contamination in final products that would preclude the delivery of such a product without even knowing the sterility tests. We do ask that those are done. As far as the pyrogen or endotoxin test, as I think I alluded to before, endotoxin may be used during the investigational phase in lieu of doing a pyrogen test. The pyrogen test that is present in the Code of Federal Regulations is actually injecting a subject into a rabbit to see if it gets a fever, and that is a little bit unwieldy. In fact, testing for endotoxin can be done very quickly and should be available before lot release and also, in fact, it may be possible, upon application to sponsor, to use endotoxin as an alternative to the pyrogen test, depending on, again, the application, the sponsor, the product and so forth.

Let's talk a little bit about viruses since that seems to be a main focus. One major kind of assay that we ask be used to detect viruses is a culture assay *in vitro*. We ask that the products be co-cultured with a panel of indicator cells that would be able to pick up different kinds of viruses. We ask that a cell line representative of the source animal species be used, a cell line representative of the animal tissue type being used, and a human cell line be used for co-culture to pick up viruses. Such cultures should be observed for cytopathic effects, focus formation, RT is reverse transcriptase – that is an enzyme that is characteristic of retroviruses -- changes in cell growth or unexpected changes other than those.

In addition, we ask that cultures be tested at the end of the period for heme absorption and hemagglutination with red blood cells from three different species which are tested that are also known to identify certain viruses. If there is a virus that is observed, we ask that an attempt be made to identify it using specific viral probes when available, such as polymerase chain reaction testing or immunoassays with antibodies. If viruses that have not been previously identified come up, you need to try to figure out what they are. We ask that lot release specifications be set for such tests based on available data. That is the data that is obtained by the sponsor. We also ask that, if it is feasible, to obtain the data from cell culture assays before lot release, before the product is administered, that such be done. This would be the case in particular with frozen samples. We also finally ask that assays be qualified in a number of preclinical runs. So, the first time you do your test is not when you are going into a patient.

Occasionally, *in vivo* assays in another animal may be necessary to detect certain viruses. Of concern is the activation of latent viruses. A determination of such viruses depends upon the kind of virus and the tissue type that is being used. These are just examples. For example, an expression of endogenous retrovirus can be induced by culturing *in vitro* just with nothing or by treatment with iododoxiuridine or a demethylating agent such as 5 beta cytidine, agents that will affect the nucleic acid structure. If for some reason ganglia are being transferred, then one would want to test them for activation of herpes viruses. Positive results in such tests may not preclude use, but at least should be considered in monitoring, and positive results should be discussed with CBER. One should always also evaluate the potential of the process to produce such latent viruses.

Finally, PERV, porcine endogenous retroviruses. Since these are currently a big concern in the use of pigs as source animals, and since we know that all pig cells contain PERV sequences, we have some special advice on this. We ask that sponsors evaluate, using appropriate assays, the production of infectious virus. One way to do this would be to co-culture for at least 30 days or 10 serial passages of the culture of a xenotransplantation product or the relevant surrogate. We ask that co-culture use appropriate indicator cell lines. In this case, examples are a human embryonic kidney cell line, 293, which is very popular for this use, and the swine testis cell line ST. Depending upon the cell line and the co-culture system, certain tests can be used to analyze for transfer of PERV to indicator cells, and we describe these in our guidance document. If evidence for virus production is obtained, again,

characterization of the virus needs to be performed, and this should be done in consultation with CBER.

The last level that I am going to talk about this morning as far as controlling for infectious disease transmission involves something that we don't usually think about, and that is the manufacturing facility and good manufacturing practices considerations. Facilities can play a major part in whether something is transferred into a final product, and facilities for harvesting and/or processing need to be designed to minimize such contamination. Of course, by the time any product is approved for marketing, all processes and all equipment and all manufacturing processes need to be validated, and many validations can be phased in during investigational phase. The exception to that is sterility assurance, which needs to be validated prior to the initiation of clinical trials.

In summary, I have discussed some FDA requirements and recommendations to minimize the risk of infection, and I would like to emphasize that these kinds of recommendations and requirements cover several phases of the production of a product, so that it is not something that you think about just once when you are trying to produce a xenotransplantation product. As far as the post-xenotransplantation procedure and monitoring, you will hear more about FDA's suggestions for that tomorrow.

If you want to obtain any of our documents, there are many ways, just like many levels, that one can do this. Thank you.

[Applause.]

DR. KASLOW: I think we will take only questions of clarification rather than discussion right now. Any brief question to Dr. Bloom?

DR. LUBINIECKI: During your talk, you had mentioned just now that validation is necessary. I think you mentioned that all processes should be validated. I think the draft guidance document also uses these words, that all processes should be validated. With other kinds of biological products, I believe the standard practice is that all critical processes and critical parameters for processes should be validated. Is this a different standard than is used for other biological products?

DR. BLOOM: I think you could probably read into that all critical processes. For example, you wouldn't need to validate aliquoting an enzyme but you would need to validate stability of that enzyme.

DR. LUBINIECKI: One last question. Processes such as surgical removal of organs from animals, what is your vision for how this might be validated?

DR. BLOOM: That is a good question and we haven't gotten so far as to receive organs yet. I could only speak from what I would imagine. That would be a means to be able to maintain the function of the organ and to show that that function is maintained, and a means to be able to maintain the security of that organ as far as sterility and microbiological stress is concerned.

DR. SYKES: Could you clarify how specifically you have defined what a barrier facility is? For example, does it include measures to keep out rodents, insects and other possible vectors of infection?

DR. BLOOM: Yes, it should. A barrier facility – what we ask is that a sponsor tell us what their barrier is. Then we will evaluate that. A barrier facility could be very strict with individual cages for animals or individual housing for animals. You can have similar animals housed together and then quarantined later. Yes, we do ask that arthropods, other mammals, other animals and other sources of infection and

disease be excluded from the barrier.

DR. SCHECKLER: One question as far as the similarity or dissimilarity with allograft rules, the 50-year rule particularly, is there anything equivalent in looking at allograft tissues, samples, personnel exposures, and so forth, versus xenografts, since the model that you are using, the 35-year HTLV model, would be equally of concern, I would think, in allografts?

DR. BLOOM: Frankly, I am not entirely sure if there is such a recommendation in place for allografts. However, let me say that with xenotransplantation products, our concern is that there may be an infection that could conceivably arise that we really can't identify, we really don't know about, and we won't be able to know about unless we go back and look at early samples from early on. If those aren't saved for a sufficient amount of time, we won't be able to look at them. There is some difference in that regard as far as xenotransplantation archiving. I don't know, maybe Dr. St. Martin or Dr. Chapman know about whether or not there are any similar regulations in allotransplantation or recommendations.

DR. ST. MARTIN: There is no recommendation or requirement for storing or keeping records for any specific length of time. It all depends on the length of the life of the recipient. The recipients are followed up to a certain point after their death or the failure of the graft, but there is no requirement for any long-term storage of records.

DR. MAHY: Could you just technically say where this is going to take place, this 50-year period? Is it going to be a central bank of material at FDA or is it going to be distributed?

DR. BLOOM: The location of a central bank, in fact, the creation of a central bank is something that the Public Health Services, the Department of Health and Human Services is considering at the current time. Until such a place exists, FDA asks that sponsors maintain the banks, but still have specimens that would be accessible by the Public Health Service and for patient care.

DR. SALOMON: One of the things that is real interesting about this particular discussion of a 50-year data base is parallel to something that is very controversial right now in front of the biological response modifier advisory committee in the context of gene therapy, where we have also had discussions, rather heated actually, on how long a sponsor should maintain records. The real problem is who is going to pay for it. An NIH grant is four to five years. One of the major concerns we have, biotech companies, of course, come and go. Maybe big pharma companies are going to be around for 50 years, or even 25 years, but the time frame on most everything else going on in these cutting edge areas of biotechnology are much shorter. If institutions, for example, where independent investigators are doing what they are supposed to do, which is pushing into the gray areas of science, are suddenly made responsible – in other words, the individual investigator, for example, could not get an NIH grant to do a clinical trial in gene therapy or a clinical trial in xenotransplantation unless the institution was willing to guarantee 50 years or archival support to that investigator. One doesn't have to be a rocket scientist to do the math. That would be pretty much the end of this kind of an academic based initiative. I find this -- I don't have any good answers for you, not for gene therapy where I have been really torn up about it, and not now for xeno either. It is really not something to take very lightly.

DR. KASLOW: I have a feeling we will be coming back to this. Perhaps we should postpone the rest of the discussion and move on to the next speaker. We will take the second topic under this general heading. Dr. Michael Swindle, from the Medical University of South Carolina on animal husbandry techniques in swine to minimize the risk of transmission of infectious agents.

Agenda Item: Animal Husbandry Techniques in Swine to Minimize the risk of Transmission of Infectious Agents.

DR. SWINDLE: My charge today is to talk to you about husbandry effects, so that we can protect people from diseases of the pig, and also for protecting the pig from becoming inadvertently infected. I am limiting my remarks to swine because I think everybody agrees that primates are not going to be conceivable as an organ donor for humans on any major scale.

I want to point out, one of the things when you are designing facilities is, it is going to depend on the type of pig you use. For instance, a domestic pig reaches a weight of about 240 pounds in four to five months, and miniature varieties may only reach 40 pounds in the same period of time. So, caging differences and sizes will vary. Most likely, we will be dealing with transgenic donors, so we have to deal with those particular infectious issues. It is possible to maintain them pathogen free.

The most important thing has already been addressed, and that is that we have pathogen-free breeding stock maintained in isolated facilities at about a safety level two. That we have a sentinel animal program is probably the best way to monitor a herd. In other words, you have a room full of animals, and you have some animals that are just there for taking serology and doing complete necropsies and things of that sort. Those are time tested methods that have been used in rodents for many years, and it is a pretty safe bet that, over time, anything that occurs in the sentinel is going to occur in the rest of the herd.

Now, in addition to the diseases that have been discussed, I want to mention two other things in pigs, lest we forget about them. One is that pigs have congenital defects, especially in the cardiovascular system, and ventricular septal defect, patent framing ovaes, things of that sort, do occur on a regular basis in pigs. So, if you are using certain organs, you should consider that. The other thing is there are neonatal neoplasias that occur in swine, embryonal nephromas – that is tumors of the kidney. Also, there is a heritable malignant melanoma that occurs in some strains of pigs as well. There are other things for the producer to consider.

Now, in addition to using a good source, it has been pointed out that the animals should be vaccine and food additive free, certified food, bedding and water. They will most likely have to be cesarean derived, your initial stock. So, how do you do that? There are going to be gnotobiotic types of transgenic animals. You have to raise them in isolator units. There are commercially available ones, but most likely people would be manufacturing or custom designing their own in their facility. Basically, you are talking about a bubble or a transparent box where you are working on animals with gloves in a facility that has been presterilized with peracetic acid, which is more or less the industry standard for doing gnotobiotic animals.

Now, the sow delivers the pigs by cesarean section and is killed. So, the pigs are orphans and they are probably going to have to be fed sow replacement formula, which is commercially available, and not foster nursed on another mother, which is the standard in rodents, because of the potential of transmission of disease. Then, if you raise animals this way, you have to provide them with the normal bacterial flora in order for them to properly digest food, which is some sort of difficulty to deal with, another technical difficulty. You can move animals out of these types of units into a regular barrier facility in about two to three weeks, to where they can start eating on their own. That is early weaning. Three to four weeks is probably a safer bet in most cases. The sentinel animals are put in the room with these animals at the time that they are moved out of the barrier, and this is where you do all the sentinel screening which we have alluded to before.

Now, the donor testing has been discussed enough that I am not going to be redundant about it. Again, you can't do it in this type of situation. Now, this is a regular commercial hog-producing setting, quite standard, screened-in windows and more or less open types of facilities. What we are talking about here is NIH guide, AAALAC accredited types of facilities, and detailed standard operating procedures. Standard operating procedures, in my experience, have to be fairly detailed, because people change, and legends become fact unless you have got SOPs that people are signing off on that they are doing. You have to certify your husbandry conditions. So, it is our experience that because somebody said they did something ain't necessarily so. So, we have check lists on almost all of our husbandry procedures, and you have to keep records of this, of course.

Now, the environmental standards that are out of the NIH guides and are generally acceptable -- this is heating, ventilation and air conditioning -- it is a positive pressure room, so you have got positive pressure keeping things coming in from the corridors. Ten to 20 air changes per hour would be preferred to keep down ammonia levels and things, hepa-filtrated air, so that nothing is coming in and nothing is going out. Temperature range of comfort for pigs is fairly wide. We have a tendency, on non-surgical pigs, to go in the lower range, around 72 degrees. Post-surgical, though, they do much better if they are in the higher temperature range, upper 70s. They can stand a wide range of humidity, 30 to 70 percent kind of standard. You should have back-up power, because basically you are putting all these animals in a sealed environment. It doesn't take long, once the electricity is gone, for these rooms to get steamy and stinky and ammonia levels build up and things of that sort. Then another thing, in long-term breeding situations is, pigs are social animals and need some form of socialization.

I will use our facility as an example here, and at least to me this doesn't come out so well. Basically, what we have is one floor of a building which is self contained. We have our operating rooms, animal prep rooms, surgeon rooms, diagnostic laboratories, x-ray and cath labs, showers and lockers, and then the animal rooms on another side. In fact, in this animal facility, you will need multiple rooms, because this is an all in, all out procedure. You should not be adding animals to a room that already has animals in it, because that breaks your barrier-type facilities. In the case of rodent producers who have very intense, closed barriers, most of their breaks are probably related to animal care takers bringing things from home, on their skin and on their clothing. Most of the breaks are going to be related to people transmitting things to pigs within these facilities, and probably not fruit bats coming in through the air conditioning.

This is what a room looks like, more or less. These are the cages, which I will give you an in-depth look at. You have got to have a way to wash your hands. These are sealed containers. These are concrete block walls, epoxy seals. These are seamless epoxy floors.

This is the back side of the cage. This is a gutter system, and there is a slope back to the gutter. We use flushing gutters, where we flush them like a toilet, where you can flush things down the drains and have sealed filters. We have pre-filters to keep dander and things out of the air-handling system. Then, we have used a variety of caging styles over the years. This is one that we have abandoned. This is galvanized chain link fencing and plastic coated floors. Now, one of the things about a pig is that, even if you get the slightest little tear in this plastic, the pig will strip it clean overnight. If they can find a hole or a loose part in your cage, they will take it apart for you, just for fun.

Now, this is the system that we have been using for five years for multiple systems and we found this to work very well. These are stainless steel, fiberglass slatted floors, with grit in the floor. Now, why is grit important? Pigs have hooves. If you put them on soft-type flooring, they won't wear their hooves down, and you will have to bring in a farrier to trim hooves, which is no picnic in pigs. We have automatic watering, and food dishes have to be attached to the side of the cage, because anything you

stick in the cage immediately gets stomped, thrown, rooted around. We have been using these teflon balls, mainly, for them to play with. They like to root. They throw things around. They like to have contact with their other species and we have some split pens with vertical bars. Pigs like to go snout to snout to greet each other and like to see other types of animals. You can house them alone in single caging instead of in groups and they still get their socialization through the bars, in our experience.

Now, this is a corridor, and the corridors are basically built to the same standards as the animal rooms. You should be able to walk into any of these animal facilities with a hose and hit any part, floors, ceilings, walls, and have it impervious to moisture. So, that is the basis on which you work. Your operating rooms and collection rooms need to be working under full sterile technique and ceiling-mounted polymer -- ooh, that was a \$500 bulb and we don't have a back up. From here on, I will just go ahead and give my talk without slides to keep us on time, because I have shown the major facilities.

Basically, for cleaning and disinfection, you pressure wash these rooms and you need to fog the rooms. The standards for fogging rooms to disinfect and sterilize them are either chlorine dioxide solutions or formaldehyde. Obviously, you don't want to be in the room when you do this, but this is very effective when done multiple times. Either chlorine dioxide or formaldehyde can be used interchangeably or alternated. They are quite caustic and they will eat up the equipment in the room. So, you have to be careful about what is left in there. In between, we use quantinary ammonium types of disinfectants. That is a fairly broad spectrum sort of thing. In addition to that, to ensure quality control on our sterilization and our sanitization procedures, we use what is called RODAC plate monitoring which is where you take a microbiological plate, you touch it to various surfaces, and then you count bacterial colonies after you disinfect them to see if it is effective. You periodically find out that it is not. So, it does come out as being useful.

Essentially, the facility standards are what I told you. Biosafety level two is probably a good design phase, using the Public Health Service standards. If you are using the NIH guide, then you are going to be AAALAC creditable on facilities. The program is a large part of AAALAC accreditation. You must define what organisms you want to keep out and then design the facility and then the standard operating procedures to meet that goal and you must be absolutely definitive on what your husbandry standards are and your personnel training and all those issues, because it is people who are going to create the breaks in these rooms, not other pigs. Thank you.

[Applause.]

DR. KASLOW: Thanks, Michael. Clarifications or questions briefly before we break?

DR. ALLAN: I may have missed this, Mike, but I notice that here the pigs are singly housed. Is there a reason why they can't be housed in groups?

DR. SWINDLE: No, they can be housed in groups. The reason we singly house them is most of our pigs are survival surgery candidates. Pigs are cannibalistic. If one has blood on him, the other pig will eat him, and basically you cannot house them together in that situation. We do group house small animals and animals presurgically. One of the problems with that is that pigs fight and create their pecking order. If they are not socially compatible when you put them together, they will soon sort it out, but there will be pigs without tails and ears and everything else. In our situation, we singly house these animals unless they are littermates and have been born and grown up together. Otherwise, you will get wounds on pigs.

DR. ALLAN: Would you recommend that they be singly housed or group housed?

DR. SWINDLE: If I were doing it?

DR. ALLAN: Yes.

DR. SWINDLE: I would do it in the split cage format, like I showed you, where the pigs still get some socialization, can see, smell, touch each other, but I would singly house them just because of the injuries. That is another thing about the floors that we have there. We have not had a single animal gets its hoof caught or injured on that particular type of flooring, where you do on a lot of other types of surfaces. If you are using surfaces that get wet and stay slick, pigs can fall, break legs, hurt themselves and things like that. They need traction, and that particular system has worked best. We have used just about everything there is over the last two decades and that is what we came down to.

DR. ENGSTROM: Michael, a question about sentinel animals. You mentioned how important they are. How are they selected, and do you have criteria? For example, do they have to be the same age, same gender? In terms of the number, how many should you have?

DR. SWINDLE: In terms of rodents, for instance, you want immune competent animals. So, if you have a colony of nude mice, your sentinel is not another nude mouse. In pigs, it probably should be a similar situation, a cesarean derived animal, but not an immunocompromised type of animal, but one that has come from a cesarean-derived line, so you are sure it is clean before it goes in there.

DR. ENGSTROM: It should be the same age as the others?

DR. SWINDLE: Yes, they grow up with them, basically. You would probably be doing sampling in large animals about every three months with those sentinels, and maybe drawing serology more frequently than that.

DR. KASLOW: If there are no other questions, we are going to take a one-hour lunch break and return at 1:30, in effect, and we will resume with this section again at 1:30.

[Whereupon, at 12:30 p.m., the meeting was recessed, to reconvene at 1:30 p.m., that same day.]

AFTERNOON SESSION

(1:40 PM)

DR. KASLOW: Ladies and gentlemen we are going to convene again. We need to maintain our pace. We will begin the session with the third of the presentations in the last section with Dr. Anthony Lubiniecki from GlaxoSmithKline speaking to us about lessons learned about viral screening from other biologics.

Agenda Item: Lessons Learned About Viral Screening from Other Biologics.

DR. LUBINIECKI: Thank you, I would like to begin as Dr. Potts said a little earlier by noting for the record that my employer, GlaxoSmithKline does not do research or development in xenotransplantation, not directly nor do we have business dealings with other organizations that do that work. So, I have no

activity in this field, but I did want to spend a few minutes talking about some of the things we have learned in the last few decades about other biological products that probably have some relevance to xenotransplantation. So, I want to talk a bit about some of this history, review some of the testing methods, talk about some of the prevention methods and some other tools to prevent viral contamination and finally, to try to put this in context for how one might apply some of this to xenotransplantation.

The early history of biological products which dates now back several centuries is that there were lots of problems and in general just to put this in perspective there was lack of what we know today as aseptic handling technology. There was lack of good manufacturing practices. There were pictures of famous scientists making antidiphtheria serum 100 years ago standing in their Sunday-best suit in the GMP barnyard bleeding the horse, and this was common practice.

There was lack of adequate test materials, adequate test methods, especially for viruses and so a lack of process validation or other controls that today we would consider essential to make these products in a safe manner, but by the 1970s the situation was greatly improved. We now had the institution of GMPs, good manufacturing practices. We had developed the notion for individual cell products. We had the ability to create cell banks and to characterize these cell banks for whether or not they were contaminated by viruses and other things.

A good deal of modern assay technology was coming along and as mentioned by the last speaker HVAC technology allowed us to have air that was pretty clean. Just to give you some examples of some of these horrible problems that we had the very first smallpox vaccines which were actually cowpox and they were basically in those days there was no manufacturing process. You found the local milkmaid who had the lesions and you took a small amount of pus from a pustule and you inoculated a human and there were outbreaks of syphilis that were noted from this practice if you chose the wrong person.

In the 1950s a great medical advance was achieved by the creation of IPV or inactivated polio vaccine by Jonas Salk and a number of others and in the 25-odd years that people continued to look into monkey kidneys there were approximately 60 to 80 new viruses that were isolated from monkey kidneys. Monkey kidneys were used as a source of the cells that one then used to propagate polio virus before it was inactivated. So, this was I think an interesting lesson that until you look you really don't know what an animal species has in the way of viral contaminants and in this particular case the science of virology was sort of evolving at the same time and so it may not take 25 years to find out how many viruses are in pigs, but it probably won't happen in an afternoon even though our technology is a lot better today.

At any rate dozens of previous unknown primate viruses were discovered, and it was one of the most fascinating with the discovery of simian virus 40. As you might imagine it was the fortieth new virus to be isolated from rhesus monkey kidneys, and it had the unusual property of both not causing too much cytopathology in rhesus, the species of origin but, also, being resistant to formaldehyde and as a result even though the polio virus in the vaccine was inactivated by formaldehyde the SV40 was not. It was subsequently found or around the same time found that SV40 was capable of infecting human cells and also causing some biological changes in those human cells. So, by the time this was all realized approximately 10 million Americans who were basically my age or older and another 10 million or so in Europe were in fact inoculated with live SV40 as part of their Salk polio vaccination.

Another example is the transmission of hepatitis viruses via blood products prior to the availability of analytical technology to look for hepatitis viruses or antibody to hepatitis viruses in blood which started in the middle seventies. There were tens of thousands of cases of hepatitis that occurred every year in the US alone in people who received blood products and plasma derivatives.

There were other examples of incompletely inactivated viruses and toxins and of course hundreds of known cases of microbially contaminated products in the early days, but things have moved on from there. We have learned a lot of lessons. We have created some great new technologies and we figured out how to manufacture products which are much, much safer to use. We know now that blood and tissue as a source of materials for biologicals contain multitudes of infectious agents as we have heard this morning, and while safety improved as test methods improved we, also, now realize that testing alone is usually inadequate to assure the lack of contamination. You basically cannot test quality into the product. It is true for cars. It is true for other biological products, and it will probably also be true for xenotransplantation products as well.

However, we, also, now know that prevention of contamination can be a very effective tool to assure product safety when applied to a variety of things that go into the product such as the raw materials, the environment in which the product is made, source materials organ for organs and cells and also for the production staff. More recently the use of characterized cell banks has been widely used to prepare many viral vaccines and all purified protein products from recombinant technology as practiced in cell culture as well as monoclonal antibody technology. In this case the cells are cryogenically preserved until use and they can be extensively characterized for endogenous and adventitious agents of microbial origin and viruses, and if one looks at the last 15 years of the use of such products commercially it is possible to say that purified protein products in some vaccines are certainly capable of being treated during processing to inactivate or remove putative viral contaminants.

One of the things that has arisen in these last 15 years is the concept of the integrated use of a variety of methods. In a way it is sort of like peeling an onion. No one method by itself is foolproof but if these methods are integrated in a constructive and deliberate way one can actually achieve great progress and safety by using multiple overlapping layers of protection. So, for example, the integrated use of characterized cell banks and process validation concepts together with GMP controls over source and raw materials controls on production facilities and staff combined with an appropriate testing program can provide a high level of assurance of freedom from microbial and viral contaminants for cell culture-derived products and it is, also, true that there is no documented evidence that any of the millions of recipients of these recombinant products or monoclonal antibody products or the hundreds of millions of vaccinees have been infected by any viral contaminants transmitted by the products.

Now, I am not going to try to tell you that the absence of evidence equates to the absence of risk. That is not true, but I, also, think it is equally true that certainly for those receiving the therapeutic products, most of the recombinant products or the monoclonal antibodies are patients who are in general quite ill and receive a high level of medical attention and if millions of these folks have received these products and not been found to be injured by the product I think that it speaks very highly on behalf of the methods used to prepare those products.

However, despite the use of some integrated control systems to prevent contamination there have in fact been some isolated events that have occurred and that these events were, in fact, found or detected by various kinds of virus screening assays. Among the more common are the use of contaminated raw materials, typically from fetal bovine serum where a number of reports of the isolation of bovine papillomavirus as well as an orbivirus related to retroviruses have been found from serum and also porcine parvovirus from trypsin. So, these are raw materials that may be contaminated and require some level of surveillance to make sure that you don't use those materials.

The use of contaminated source materials has, also, been a problem. In the early days in hybridoma there

was one lab where several of the workers actually contracted LCM from the mice that they were using to prepare the hybridomas. There have been a few GMP failures. There was one particular incident where human rhinovirus was isolated from a vaccine pool that was not supposed to be rhinovirus, and it was linked to basically environmental failures and basically the staff contaminated the material.

There has occasionally been incomplete or inappropriate testing of cell banks. There was a product whose cell bank was tested many, many years ago before testing for retroviruses was common and in fact when the cell bank was later tested it was found to have been contaminated by an infectious retrovirus of a totally different species, and there have, also, been several outbreaks of MMV or what used to be called minute virus of mice and now is probably more commonly known as a murine parvovirus. This appears to be associated with truly large-scale cell culture and it can be because the virus is extremely resistant to inactivation, it can be very difficult to get rid of it. This has not been found very often, but by my count at least five different organizations have run into this in the last 20 years. So, it is a problem that is certainly out there.

Very few batches give positive results in properly controlled processes. The one firm with the most experience here seems to be able to report that about one batch of 1000 will have this problem, and it is not certain where the problem comes from. They have exhaustively searched their raw materials and so forth, but it can be very difficult to find some viruses especially when they are present in nature. This virus can be spread by both the fecal as well as from the urine of infected mice, and as we all know there are lots of mice in the world. It, also, can give rise to testing problems because almost all testing is based on the assumption, tacit or otherwise of homogeneity, that is any sample adequately represents the universe being sampled, but when the virus is coming from discontinuous sources such as feces it isn't clear what a sample tells you. So, it is a very difficult problem to attempt to control, but I think the important message though is that while viral contaminants are rarely found they are found in fact by virus testing in the process or the product and that existing control systems prevent the release of these batches of product.

Despite the use of integrated control systems of contamination, I am sorry, I went backwards. Some of the kinds of viral assays that have been used for these purposes include biological assays whether *in vivo* or *in vitro* and in general as a group some can be quite powerful but inevitably one cannot detect all possible viruses in a single biological assay. So, this necessitates the use typically of multiple biological assays, frequently involving different species of cells. Sensitivity is often excellent. One can frequently go down to about one infectious unit per sample volume, but if the contamination is less abundant than that the method will frequently not find the agent one is looking for. They are not usually rapid. It can take weeks and in some cases months to run these kinds of assays especially when the amount of virus is low. The reproducibility can be excellent if the level of contamination is high, but as the reproducibility approaches or as the level of viral contaminants gets very small the reproducibility can be quite poor.

One of the sayings in viral testing is that a negative result really has not meaning. Only a positive result has meaning because there are a number of possible interpretations to a negative result. Even employing multiple biological systems there is limited assurance of detecting virus if present, and these are frequently used for lot release assay of purified protein biologicals and viral vaccines but they are quite time consuming. There are, also, immunochemical tests for viral antigens, typically viral proteins. These are nowhere near as sensitive in general as the biological assays but they are quite rapid, quite reproducible and quite specific but in general they are too insensitive for lot release assay use and they are impractical for a broad range of possible viruses.

A newer technique which is commonly used is PCR for viral nucleic acids. These are extremely specific,

quite rapid, quite reproducible under controlled conditions and they are almost as sensitive as biological assays but not quite as sensitive as the best of the biological assays. They can be used as screening assays for harvestability of cell culture fermenters, in other words as one gets close to the time when one would harvest a fermenter for a given product if one has specific concerns about specific virus or viruses that you can identify and for which you have probes you can conduct a PCR reaction and probe for the presence of that specific nucleic acid sequence, and assuming it is negative then you can go ahead and harvest the fermenter with a high degree of assurance that you won't contaminate the purification columns with a contaminated prep of product.

So, this has been introduced over the last 5 years or so, and it is quite a useful test, and it is in fact the way one deals with one of the 1000 batches of fermentation fluid that might be contaminated with murine parvovirus. However, by definition PCR is so sensitive it cannot detect the presence of a virus whose nucleic acid is not represented in the probe. So, the good news is one has complete specificity, but the bad news is one has to know exactly what one is looking for.

If one looks at the early history of cell therapy, cellular therapies as have been practiced over the last 20 years or so there have been a few things that have happened which are perhaps worth noting briefly. There was a study of autologous cell therapy which used essentially the same kinds of cell cultivation methods as are used for the purified protein products and what this showed is that one should expect to have the same or similar risk factors because one is using the same technology, and it also may require analogous control approaches.

The incident specifically was the transmission of hepatitis A virus by lymphokine activated killer cell therapies that were prepared as an autologous therapy from the patient's own lymphocytes which were then propagated in the presence of IL2 and reinfused into the patient. Unfortunately the cells were propagated in a human serum which was contaminated with hepatitis A and this gave rise to a number of cases of liver disease. So, this shows that the opportunities for contamination certainly exist in cell therapy which can become safety issues.

So, what does this mean for xenotransplantation? Among the things that it means is that viruses and certainly some other microbes will be present in source materials and they will be present in the raw materials, and they will be present in the production environment. So, for example, when we use non-human organs and tissues as source materials there will be viruses there. If we use non-human cells as feeder layers for human cells or as transplantable cells in their own right, there will be the possibility of adventitious or endogenous contamination. The reagents that we use to grow these cells have the potential for contaminating the product and of course the production staff do as well. So, it would be logical to expect that xenotransplantation products might have a potential quality issue due to the risk of viral contamination.

I won't spend too much time on this because we have already discussed that. Xenotransplantation products in general are going to be living cells or living tissues and so what this will mean is that you cannot undergo virus inactivation procedures with living cells. So, those kinds of opportunities to control the problem probably won't exist here. However, it might be applicable to some of the reagents that are used to grow the cells that would be used for xenotransplantation as an example.

In general, also, the product must be used quickly. So, we cannot wait around for the long-term biological test results on the product itself before we use the product and similarly with organ-derived materials they need to be aseptically removed from anesthetized donors and used without any product characterization. So, the risks are essentially the same as for human surgery. Having said this, even

though some tools that we have seen in the past were useful, it may not be appropriate for the control of xenotransplantation products. Others that haven't been used for purified protein products may in fact apply and may even be useful. Michael Swindle spent a lot of time this morning talking about how one can do animal husbandry. So, I won't spend any time on that.

Cells and tissues which are cultivated *in vitro* can be cryopreserved and screened for viral contaminants and other kinds of contaminants. There are ICH or International Conference on Harmonization documents as well as documents from the FDA and a number of other regulatory authorities that give very clear guidance on how one can do this. One of them is ICH Q5A. There is also Q5D and a host of other documents. Similarly for established or continuous cell lines cultivated *in vitro* the same approaches can be used and for all cells and tissues cultivated *in vitro* I think it is possible to also look at PCR techniques for screening for selected viral agents just prior to harvestability to provide some additional insurance.

In the processing environment one can minimize the amount of contamination acquired from human environmental sources by minimizing the use of open processes so that one should either use closed or contained vessels like fermenters where it is appropriate or if that doesn't work for the technology then consideration of some sort of barrier containment devices basically biological safety cabinets if it is compatible with the technology for the product. This technology has been used for the last 20 years or so to provide an extremely high quality and reproducible aseptic environment and it is now a very proven technology for a number of applications including sterility testing, aseptic pharmaceutical drug processing and roller bottle cell culture. Most of the erythropoietin made in this country comes from such a contained process using roller bottle cell culture of recombinant cell lines. So, this technology is quite good and provides sterility assurance that is very, very high.

The final comment I would like to make is that I think it is important that we use the tools wisely. It is not possible or prudent to do everything imaginable. It is just not a sensible thing to do. However, that doesn't mean we should be imprudent and go out and bury our heads in the sand either. We should do things that are reasonable and prudent to do.

Not all control methods are compatible with all technologies for making products and not all control methods add value. As an example, several decades ago when the first recombinant product from cell culture was being developed tissue plasminogen activator we were confronted with a rich scientific literature because the enzyme had actually been around for quite a while, and when we did a literature screen we found 120 different assays that could be performed on tissue plasminogen activator, and this was daunting because some people thought the QC testing for this product should be every conceivable assay. When the numbers got to be 120 it just clearly was not possible.

So, what we did was we basically sorted the methods by what the methods actually measured and then we asked ourselves what do we actually need to know about each batch of product in order to be sure that the product will do its job, and when we did that we found that about 20 assays would measure everything that was worth knowing about the product and the other 100 could be disregarded.

So, this is a very practical way of approaching things, but it is used widely I think on most recombinant and monoclonal antibody products, and finally reiterating the theme that it is not possible to test quality into the product one can only get quality by design, and one has to design quality into xenotransplantation products. I think that means making informed decisions for each process and product based on evaluation of the risks and benefits just as for other biological products.

Thank you.

(Applause.)

DR. KASLOW: We will take brief questions before we continue with the next two presentations.

Questions for Dr. Lubiniecki?

Okay, we will have an opportunity to comment later. We will hear next from Dr. Patience. This section is Porcine Infectious Agents; Implications for Xenotransplantation. Dr. Patience's title is Viral Safety Issues for Xenotransplantation.

Agenda Item: Viral Safety Issues for Xenotransplantation.

DR. PATIENCE: Good afternoon, everyone. My apologies for not making it this morning, but as soon as I finish this talk I am going to walk back to Boston because it is quicker than flying nowadays. I might ride a pig. That may be a little bit quicker again. Many thanks for the invitation to come here and present the work that we have been doing entitled Viral Safety Issues for Xeno. I am going to spend the majority of the time talking about PERV, but I would like to just touch on some of the other microorganisms very briefly at the start.

I think one important point which needs to be brought home is the actual source of the donor animals and I am concentrating on pigs here. These animals are all coming from designated closed donor herds. We are really talking about pigs that are not going to be ever exposed to any of the exotic microorganisms such as the Nipah virus Monangle virus and I would very much like to comment about the very small fruit bats that might be required to ever bring that into any significance. So, essentially the animals are going to be coming from defined pathogen-free source and I think we really need to focus our concerns down onto the more important organisms within that category.

With respect to the exogenous organisms, quite critical, and this may have been covered this morning, again, my apologies for not quite making it. The route of infection of animals, can there be transplacental infection or is it postpartum? In either case can the organism establish latency? For instance a little bit that I will touch on in a moment is about the herpesviruses. They tend to get a lot of focus. They are by no means the only cause that we should be looking at, but they clearly are a significant microbe, and they have implications in both this and this stage of the potential transmissions.

This is a summary of some results from both Imerge BioTherapeutics which in case anyone doesn't recognize the name is now a joint venture between Novartis and Biotransplant and is the sole xeno effort of both companies. We were looking for the presence of porcine lymphotropic herpesvirus or PLHV and also, porcine circovirus, a couple of organisms that you may have heard about already today, and very encouragingly when we were looking at fetal animals we saw very little, actually zero. We could not detect it, but I am just saying very little because again it comes back to the what is a negative comment from the previous speaker, and as the age of the animal gradually increases, I hope you can see that okay, it is not showing up to well for me from here, the incidence of infection actually increases as you would expect.

Barrier rearing of the animals drops the incidence of infection way down again. So, it looks like although there potentially is some passage of the gamma herpesvirus in this case, there are certain animals that you can isolate which are negative, and it is a matter of logistics to actually use those animals for a breeding

program. The porcine circoviruses, again, very encouraging results, animals age 3 to 18 months reared under conventional isolation conditions showed an incidence between 48 and 88 percent depending on which type of virus. Conventional rearing early on no incidence of virus. So, again it looks like the exposure is happening postpartum there.

What is an endogenous organism? Let us get focused onto PERV here. What I want to make clear is endogenous organisms are distinct from ubiquitous organisms. I still get asked this quite a lot. Endogenous organisms are carried by an animal as part of their natural germ line DNA. Essentially for pigs we are limited to the pig endogenous retrovirus, and this is the beast that we in organ research and a lot of people have been researching. This is a classical, C-type virus particle structure. The actual outside of the virus is particularly crucial because that controls what type of cells that it can infect, whether it can infect human cells, whether it can infect pig cells.

We actually think that that part of the virus, and we have got some quite nice data is very, very fragile, and what we are wanting to look at now is the effect of removing Gal sugars which have been mentioned as sort of natural immunity to PERV and whether it will actually disrupt the envelope structure even further such that particles from knockout pigs may actually be less infectious. So, that is an ongoing project which we are working on at the moment.

Apologies to the virologists out here, but this is really a schematic of a virus particle, the envelope sitting on the outside which is encoded by this gene down here, the env gene. The gag encodes the core of the virus and the pol, the enzymes which are associated. I will come back to those points a little bit later in the molecular analysis of the transmission assays that we have been looking at.

All vertebrates have endogenous retroviruses. As I said earlier they are part of the natural germ line DNA. Thus, pigs for instance inherit them. They don't go around catching these viruses as part of an infection. They are actually very stable genetic elements. Now, when we first published on the infection of human cells the fact that the PERV were endogenous and quite widespread made us all think this is going to be very difficult to overcome, but I think with the really quite exciting results that our laboratory and others have been generating, I think PERV is really turning itself into an issue which can be overcome, and I think there is a real mind set change required here. Just to illustrate that not all endogenous viruses are bad, everyone in this room, approximately 1 percent of your DNA, my DNA is made up of ancient endogenous retroviruses, exactly the same with the pigs and this is where the PERV issue really came from to start off with.

A very simple schematic of the retrovirus life cycle. Normally you get an infection stage here which is followed by the virus integrating in the DNA of the cell and then virus particles are released to go on and initiate further infections. What happens in the case of the endogenous viruses is that you can effectively forget this part here. The virus is already present in the germ line DNA and it is a production effect, and what we are looking at is whether the virus particles which are produced can go on to infect other cells.

A point I would like to bring home here is that endogenous retroviruses have no natural pathology in their natural host at least because if an animal was subjected to an endogenous virus which had a pathogenic effect over the millions of years of evolution the animal would be placed at a negative advantage or even a disadvantage and therefore it tends not to be, that type of virus tends not to be represented in the germ line of animals today. Indeed some of the endogenous retroviruses are actually essential to the host. We have viruses which are essential to salivary amylase(?) for instance, expression for us and also physiological functions, potentially the correct function of the placenta, for instance in humans.

Obviously what we are all interested in here is the possible pathology if the virus A jumps species and B, replicates and C, causes a pathology, so many steps in there.

I would just like to point out that the endogenous retroviruses of pigs are related to exogenous viruses, i.e., viruses which animals catch. They are related to the leukemia viruses. The PERV which we all hear about and gets the focus of the PERV research is related to the leukemia viruses. There are other PERV that the pig carries which have no replicative ability which are related to for instance the mouse mammary tumor viruses. What I think is important is that endogenous retroviruses related to the lentiviruses whenever people say, retroviruses, they think of HIV in a lot of cases. Endogenous viruses related to HIV have never been identified in any species.

So, just to give you a feel, for the non-virologists this is essentially a family tree of viruses. We have PERV sitting down over here with the leukemia virus related C-type viruses, murine leukemia virus and gibbon-8 leukemia virus. There are, also, a few PERV over here next to the mouse mammary tumor virus, nothing near the HIVs or near the HTLVs either. It is very, very limited to a little close-knit group on the left hand side there.

As I say, the PERV that we are interested in are stable. They are present at approximately 50 copies per cell. Because there is a selection away from viruses that can grow, the vast majority of those 50 copies are all going to be defective, and interestingly and very, very encouragingly because animals inherit different patterns of PERV from their mother and from their father, animals can be shown to show polymorphisms of PERV. So, one pig which has a particular collection of PERV is not the same as another animal which has its own unique collection of PERV allowing us to examine individual animals very closely and to select those which we think are going to be the most appropriate for the donor herd deriving.

A point which I would like to make, we have got this very stable genetic element, and I think actually that stability we can turn around and use to our advantage to breed animals which do not produce PERV which grows in human cells. This type of inbreeding program has been very, very effective in mice, exactly the same type of inbreeding program that we are performing in pigs, and you can breed mice which do not produce viruses which infect human cells where other very, very closely-related mice do. Now, that stability gives us control, and I think that is really something we need to make the most of for the entire field.

So, we have first inbreeding programs that we can use to combat the PERV issue. Secondly, we potentially have knockout technology. Because the PERV are integrated into the genome of the pig they all have unique flanking sequences of host DNA, non-viral DNA. Once you identify those and once the technology has developed knockout genes in pigs it will be possible to knockout particular crucial loci copies of the virus if you cannot do it with typical breeding patterns or programs.

For basics really PERV is produced by both pig cell lines and primary cells. A point which I will come back to in many cases here is I think the majority of the research performed to date has been done on pig cell lines, and I think we are starting to get indications that the field needs to go through a re-education again such as we did when we were researching HIV in that a lot of the tissue culture cell lines which are used do not reflect what is in the real pig.

Now, there is a lot going on in that now, and I hope we can come back and present some data here, but I think a lot of the data needs to be re-examined. Nevertheless there are three infectious PERV families from tissue culture cells, PERV-A, PERV-B and PERV-C. PERV-C only infects pig cells. PERV-A and

B infect both pigs and humans, and essentially what we have done is develop tissue culture assays that emerge to compare the relative productions of these families of virus to compare the ones which can infect pig with the ones which can infect both pig and humans.

This is very simplistically what happens. You take your stimulated pig cells which are going to produce the PERV because it is present in them naturally. You stimulate them to hopefully produce as much virus as possible and therefore make your assay as sensitive as possible. It is a very simplistic assay where the cells are then split into two lineages really. One side you mix in target human cells and then leave it in extended culture and look for virus and in the other side you mix in other uninfected pig cells and look for production of virus.

The actual herd which I am going to describe here which we work on at Immerge was derived by David Sax at Mass General Hospital and has been inbred for over 25 years now a very complex breeding program going on. David maintains the herd with respect to doing very interesting tolerance work and transplantation work based on the MHC. We have been looking at it obviously from my personal point of view from the lineages of pig with respect to their virus production and transmission.

This is a summary of the results that we have got to date. You needn't worry about the various haplotypes or pig lines that we have used here. What we have done is as I said split the transmissions into either a pig cell trying to produce virus to infect pig or to infect human. The take-home message here is that every single assay that we have performed here we get productive infection of the pig cells. We get an incidence in some lines of pig up to about 50 percent, some a little bit lower and quite critically here we get a couple of lines where the incidence is very, very low.

It is only very recently that we have actually identified these three here, these three animals in the lineage and very, very interestingly that is from one father and two offspring of the father, again, pointing straight towards a very controllable genetic reasoning behind the production of PERV and the production of infectious virus. The one animal in the G line that we present there was a very old animal which was never used for breeding and is no longer represented in the ongoing animals which were the other nine out of 10. So, again, we find that very, very encouraging that a very simple inheritance is causing the ability to transmit the PERV.

You will not get quizzed on this afterwards. This is the type of breeding program which goes on. It is actually of the C lineage just to show you that very closely linked animals such as 12182, the red indicating infection can be very closely related to non-infecting animals. This was really, if I go back a couple of slides, the challenge was to say how significant are the 25 of the 28 animals that we have screened, how significant are those 25 animals, for instance, or the nine of 10. A lot of the animals although we have tested 28 animals here you can see there are actually more tests done on the animals. So, we are trying to determine how significant our negatives were and what I would just like to show here is some of the results to find out how robust the tissue culture assay is and how sensitive it is to isolate or in isolating viruses.

We have here every unit counts for a pig. We have the negative assays down the side here and the positive assays across the top. You can see here that within the miniature swine herd we have got 42 animals which we have tested once which have come up negative. We have, also, got a number of animals which have been tested twice and have twice turned negative, three times turned up negative, never with a positive and four times negative, never with a positive, and similarly for positive animals we have six animals which have been tested and always come up or a single time come up positive. We have two animals which we have tested twice. Both times have been positive, and three animals which have

been, sorry, one animal which has been tested three times has always been positive, never had a negative result from it. So, we think the assays are very, very robust and very reproducible.

We do have three animals here where we have had one positive and one negative and in one case two positive assays and one assay was a negative but again in the total number of tests which have been done here it looks like the reproducibility of the assay is very, very good and therefore we are very encouraged by the negative transmission of some of the lines within the miniature swine.

Interestingly when we look at the viruses from the animals which do infect human cells they are all recombinants. If we take the white virus as being the type of virus which infects human cells and the yellow virus being the type of virus which infects pig cells every single virus that we have isolated from the human cells which were infected are recombinants between the virus which can infect pigs and a little bit, just a little bit, the outer envelope which I mentioned earlier in that electron micrograph of the virus which can infect humans. Why the recombination? I think it is trying to tell us something and I think this is really a focus that we need to get down to. It may well be that within the miniature swine at least there are no replication competent PERV-A in the genome and therefore that recombination is absolutely required if a virus is ever going to be able to replicate in human cells.

Alternatively there may be replication competent PERV-A there which is of just such a low competence or low ability to replicate that you just cannot pick them up with the standard co-culture assays. However, those viruses will be outgrown once the chance then occurs and they recombine with the PERV-C again, the virus which can grow in just pig cells, and I think answering this question here of why we always get recombination in any virus which grows is going to be quite critical, and I think our focus will be turning to analysis of actually the pig genomic DNA again and not more tissue culture assays and tissue culture cell assays in order to get down to the bottom line in the genetic definition of why there are miniature swine which do not produce virus which replicates in human cells.

I am just going to give a little bit of an update on some of the other work which is going on at the moment. For those people who are very interested in the field there is an awful lot of molecule clones which are coming out. Again, these are coming from tissue culture cells in 99 percent of the cases and are being quoted as being replication competent. In a lot of cases I have got to say that I think it is only the exquisitely sensitive assays that we have available now that even allow us to detect these things. The classic pig cell line which we term uninfected and we use as a target for all of our assays actually produces more virus than a lot of these molecular clones. So, I think it is really quite important to bear in mind the competence of the viruses which are coming out because some of them are very, very unimpressive.

Again, I think that point is again let us look at pig cells and not pig cell lines. Other data which has recently been published or relatively recently we now know that non-human primate cells are infectible by PERV. Really I am just putting that up there to pose a question that might like to be discussed on whether non-human primates are a suitable *in vivo* model for PERV infection studies. We, also, know that human endothelial cells are infectible by PERV. Whether they are equally infectible is a completely different question.

With respect to the animal models which are going on which I believe David Onions is going to cover just afterwards I would just want to mention that there have been the publications from Diane Solomon's lab, Yarmer Deng's down in Sydney where there was an increase in the PERV DNA level in an immunodeficient pig islet model. Again, another question which I would really like to just throw in because it may fuel some interest and discussion is actually what is desired of the animal models that are

being produced. I think that is an important question. If it came up this morning my apologies, but I think it is worth bringing to the table probably again and again.

Studies showing no signs of infection, I think we have to bear all of these in mind with the comments of the previous speaker how significant is the negative result. There have been a number of publications. Cell-free virus has been put into rats. Cell-associated has been put into primates and some cell-free guinea pig data which I don't know if David will be mentioning later.

Requirement of future studies, I think really we have to look at cell-associated PERV in those types of models where cell-associated PERV are being introduced via cells to look at the various types of virus which are actually growing. It may be that in certain models which are used it is the viruses which can infect human cells which are actually infecting the animal, and that is not the type of model that you want because the virology could be completely different. Cell-free PERV assay is much easier to perform with respect to the logistics and the control of the assays but again it is how do you interpret the negative result and I think it is going to be absolutely critical for well-defined studies to be set up to analyze the type of effect.

Monitoring assays I am very briefly going to touch on. You can open probably any virology journal and these will all increase virtually every month. There are a number of immunological assays which have come forward to allow us to monitor either animal models or the human situation for signs of PERV infection, very, very sensitive PCR techniques which have come up and literally I have limited this just to the last 18 months, and there are some very, very good assays which have been around for a bit longer.

What I have been asked to mention is the antiviral data which is available. There have been a couple of papers published, one from the Imutran group and one from Walid Heneine's group in collaboration with our lab when I was back at Robin Weiss's laboratory. Both studies effectively mirrored each other's results and showed a reasonable effect of AZT in *in vitro* assays. None of the protease inhibitors as expected showed any activity. The AZT was reasonably effective. Again, another question, is it appropriate to use such a drug for treatment of an infection, potential infection that we don't know will have any pathology, just something to bring into the general forum for discussion.

Other historical data, the Xen111 study which we have all heard about back in 1999, retrospective showed no signs of infection but long-term chimerism of pig cells. The pig cells were continuing to circulate in some of the patients that were treated for quite an extended period of time up to about 8 years in some cases. Recently some more assays on porcine factor VIII, a couple of publications here again from East Coast groups, no infectious nature of either *in vitro* or again *in vivo*. A number of other negative studies. Again, it is all going to come back to the interpretation of the negatives, the Diacrin group, the fetoneuronal cells, encapsulated islets and bioartificial livers all when tested in blood turned up negative.

I think what is going to be absolutely crucial is going to be the identification of the PERV receptors or what molecule the virus uses to get into the human cells. All of these or many of these assays and studies examine PBMC or blood cells. If the PERV receptor isn't present on blood cells there is going to be no chance of picking up PERV there. So, there may be an infection going on in that individual but you would miss it. Until we have identified the types of receptors which the PERV is using the significance of these results is going to I think at least remain outstanding or still to be determined I should say.

For the future clearly I think genomic research on the PERV, let us make use of the stability of the animal. It is not going to be feasible to remove all PERV. Some of these as I mentioned earlier may

actually be essential to the animal. What I think we need to do is move towards the animal which is free of human tropic PERV or the PERV which can grow in human cells. That would certainly be a major step forward with respect to the safety.

With respect to achieving that, and I think some of the results which I have shown you may indicate we are already there with that, that that is achievable and if we are not absolutely there with those animals the technology such as the breeding program and the knockout technologies could be used to further develop the PERV-free human tropic, PERV-free pig and I think it is going to be important for mapping studies to be performed on the animals destined for use so you can actually come back from all of this complex virology and tissue culture to a genetic basis of the phenotype of a pig which does not produce human tropic PERV.

So, I think I would like to leave it there and would, also, say thank you again for the opportunity to speak and welcome any questions.

(Applause.)

DR. KASLOW: Thank you very much, Dr. Patience. A very brief couple of moments for a question or two before we go on. Comment very briefly, please?

DR. ALLAN: Yes, are you following up the studies with infectivity assays for these inbred pigs by doing like RFLPs to show in fact that you are eliminating some of these infectious PERVs from the germ line?

DR. PATIENCE: Yes, that is a good question, Jon. I think ultimately we spend a lot of time proving the robustness of those negative biological assays, and I think what we actually want to do now is to map the sentinel animals such that we can guarantee that we were not reintroducing any PERV later on down the inbreeding program. It is a good question.

DR. KASLOW: We will have time after Dr. Onions' presentation for questions and public comment. So, we will go on to his presentation now directly *In Vivo* Infection Studies with Porcine Endogenous Retrovirus: Implications for Clinical Trials.

Agenda Item: *In Vivo* Infection Studies with Porcine Endogenous Retrovirus: Implications for Clinical Trials.

DR. ONIONS: Thank you, Jim. Thank you to the Committee for inviting me to talk. I should declare two brief interests. First of all I am a director of a company that does receive revenues from xenotransplantation and secondly my University of Glasgow xenotransplantation group is, also, funded by Novartis.

Okay, that is the virus. We have seen it from Clive, the virus we are concerned about, the porcine endogenous retrovirus, and again just to pick up on two points from Clive's talk remember that this is an RNA virus. It is actually diploid. It contains two copies of its RNA genome and that RNA of course is coded by reverse transcriptase into a DNA copy or provirus that migrates to the nucleus and becomes covalently integrated into the chromosome. We will be coming back to that point because this is the point of analysis for determining whether PERV has infected human cells.

That infection may or may not be productive. If it is productive, of course, there is transcription and

translation and production of new virions. So, we can look at infection in various ways in animal models or in people. We can look for the integrated provirus or we can look for virus, say, in the plasma either by looking for the RNA genome or for the enzyme reverse transcriptase which is carried by the virus, and there are now very, very sensitive reverse transcriptase assays called RT-PCR assays that can go down in our hands down to subinfectious units because you can go down to particle counts of around about 10 to the 2.

This, of course, is what everyone is concerned about, the fact as Clive has pointed out all pigs carry a porcine endogenous retrovirus. That virus could potentially infect a human cell, may become adapted to human cells and even more unlikely, but it is still a theoretical possibility could undergo recombination with human endogenous retrovirus sequences. Most of us in the field think that is a very unlikely event and that could then become adapted and onward transmitted to other people. So, it is that public health concern that we have to be aware of.

Now, a point that was well made by Clive and I want to reiterate is that these viruses, again, this is a family tree showing PERV up here and related closely to the gibbon-ape leukemia virus and to other viruses like the feline leukemia virus. This cluster is now called the gamma retroviruses which are biologically and molecularly distinct from viruses like the lentiviruses, HIV and other retrovirus families.

I am sorry. It doesn't really matter. This gibbon is standing upside down, but this is a gibbon and the point I want to make in this slide actually is that there are examples of these genetically acquired or endogenous viruses having been transmitted to other species and when this occurs these viruses now behave like exogenous viruses in that species. In other words, what we should be concerned about in people is the idea that once the pig virus gets into people it doesn't behave like a genetically acquired virus. It now behaves like an exogenous virus and therefore we want to look at models of exogenous virus infection.

In fact, in the gibbon the virus is believed to have arisen from one of two murine species, probably in *mus caroli* at some point got into gibbons in captivity and that virus is now endemic in certain gibbon colonies, an endemic virus where these animals become persistently viremic and some of them develop leukemia. Now, interestingly gibbon ape leukemia virus as we saw is very closely related to porcine endogenous retrovirus and indeed the ultimate origin of both viruses may be from this mouse species.

Now, I want to make a point here again. Both Clive and I share a philosophy about it that this virus is not HIV. Its biology of gamma retrovirus is very different. This shows a typical profile of what happens in another gamma retrovirus infection in cats which are naturally infected by virus spread from animal to animal. So, it is an exogenous virus. In this case often what happens is that there is a temporary short-lived viremia followed by the development of antibody and an immune response. These animals become latently infected. Now, there is virus present as provirus in many cells but there is no production of virus and after several years we see disappearance of that latency. At least we cannot detect it. We believe that is because the primary infected cells are human poietic cells and that we see clonal extinction of those cells over many years. So, eventually these animals recover and this is the commonest outcome of exogenous infections in this particular species. There are, however, a minority of animals that develop this pattern, that is they don't have any antibody at all but they develop persistent infection. They shed virus from many mucosal surfaces, many secretions and they have high titer plasma viremias. So, I want to emphasize again this is not HIV. Recovery of this group of viruses does occur.

Let us now turn and look at what actually happened when people have carried out experiments to look at the transmission of these viruses in non-human primates, and first of all we are going to look at some of

the factors that might affect the outcome of these experiments. First of all, of course, these experiments are all different and very important factors are going to be do the grafts produce virus. In general most people's experience has been that both islet cells or kidneys all are expressing RNA of the virus and indeed in Dan Salomon's group it was shown quite definitively that the islet cells for instance will produce virus and this tends to be general experience that these tissues will readily produce virus. The next factor might be the length of exposure to the graft and the virus load that is actually produced and whether or not there has been any immunosuppression. We know, for instance that immunosuppression does affect these viruses.

Again, in feline leukemia virus there is an age resistance to infection such that neonatal animals are very easily infected but animals from about 6 months of age are quite difficult to infect, but if you treat an adult cat with just steroid in fact the iatrogenic levels of steroid, those animals become as susceptible as newborn animals. Similarly if you take one of those cats that is now latently infected and is recovering if you treat it with steroid you get a recrudescence from latency. You get a viremia, and they shed virus. So, immunosuppression can affect the pattern of infectivity in several ways, and this is the work of two groups, Ed Hoover over here in the States and my colleague Art Gyroton in Glasgow.

The other factor that I am sure has already been mentioned today and again like Clive I apologize if I missed it, as is known coming out of Robin Weiss's lab some years ago it was shown that the natural antibody to alpha 1-3 Gal epitopes that is present in all old world primates including ourselves will bind to and will lyse this class of retroviruses. So, the possibility here is of course that if you put just straight virus into old world primates then you might expect lysis of the virus as a protection mechanism there because of this antibody. This does not apply to new world monkeys which don't have -- these are monkeys with tails. They don't have antibody, natural antibody to these epitopes. Similarly if you produce the virus in human cells you will reduce this issue of having alpha 1-3 Gal. So, old world primates should be susceptible to infection by those viruses theoretically.

The other factors and I think a point very well made by Clive is that a lot of these experiments are now being done on tissue culture of passaged viruses, and it does change, and this is work of Caroline Wilson who is here today of course, and she showed that she could isolate viruses from porcine peripheral blood mononuclear cells and by prolonged co-culture could get the virus to grow in human cells and as she passaged it she got an increase in the titer because it was a selection against PERV-C which you remember doesn't infect human cells and also a selection against defective viruses that arise in these cultures and as Clive has already commented interestingly in Caroline's data as well the viruses that she got out were actually recombinants between PERV-A and PERV-C but containing the domains that give it a PERV-A receptor usage.

Now, I put this slide on because it is an interesting study and also because in studying that dimension later this virus amongst other viruses has been used to infect primates and the point here has been that this has been a virus that has been if you like hotted up and you hope that this virus is actually going to infect primates. So, you are really trying to use a worst case scenario and as Clive has pointed out that may not be the natural situation in transplants.

So, let us look at some of these studies. There have been a number of studies, the first of those by Vinkler who used cynomolgus monkeys with kidney grafts that lasted up to 2 weeks with quite severe immunosuppression. These animals were looked at by PCR for integrated provirus and also for antibody and none were found. There has been a more recent follow-up from the study and again with the same conclusion that there is no infection.

Another interesting study was again actually from the same group from Ulrich Martin where he put in 1×10^7 porcine endothelial cells intravenously into baboons, again with and without immunosuppression and again he was unable to detect infection of peripheral blood mononuclear cells and other tissues taken at necropsy. This fairly large study carried out by some members of the Committee here and other members by Switzer et al involved several different studies. This involved bonnet macaques which had skin grafts with immunosuppression, baboons. This involved actually both irradiation and also cyclophosphamide if I remember correctly.

There were experiments with baboons in which porcine hearts were put in with immunosuppression. These hearts didn't last for very long. They were there for a few days. There were rhesus macaque studies in which autografts were put into a variety of different macaques, some with diabetes, some with immunosuppression, some without. All of these, of course, are old world monkeys and therefore we might expect the complement inactivation system I mentioned earlier to be operative, but, also, two new world monkeys were used where we would not expect that complement inactivation to be operative. In none of these animals was PERV infection detected by PCR. There was no antibody indicating infection and response and moreover there was no microchimerism detected in these animals.

Now, this is a study that I have been involved with with Yoken Denner and I have Yoken's permission to just comment on these results. It has just been submitted for publication. This involved the use of three species, the rhesus monkey, the pigtail monkey and the baboon and in this study two viruses were used, a subgroup B virus grown in 293 cells and the virus developed by Caroline Wilson which as we heard is an AC recombinant we mentioned earlier. In this study what I think was particularly useful about this study was that first of all each of the individual animals in that study were shown to be infectible by the viruses used *in vitro*. So, what happened these animals were bled. They were stimulated with mitogens and then followed up with IL2 and then these cultures were infected. In fact, with Caroline's virus all three species were infectible and two of them were productive. In the pigtail monkeys you could find DNA in the cells, but there wasn't any production of virus. With the B virus again there was DNA detectable in all of the peripheral blood cells but in this case there was no production of virus.

These animals underwent a really quite severe immunosuppression regime and it is a regime that mimics the sort that would be used during organ transplantation and involved 5 days of precursor involving cyclosporine A and then it used a complex of both cyclosporine A, methylprednisolone and also RAD which is the Novartis rotonmycin(?) derivative. This was maintained for I believe 28 days. I am not sure of that. I will have to check that, and these animals were killed some weeks later. Three of the animals died actually due to bacterial infection because of the severe immunosuppression between 1 and 3 weeks and were also analyzed. In none of those animals was any infection detected by PCR nor was there any antibody and that was after challenge with vast titers of up to 10^9 infectious units. So, here we have a case where we have got high titer virus adapted to primate cells, no issue of complement inactivation or at least a minimal one and yet still there is no evidence of infection in these heavily immunosuppressed animals.

Let us now take a look at what has been going on in small animal models and similar groups have been involved and some of these have been mentioned. Two particularly interesting studies were from Dan Salomon's group here which has already been mentioned which I am going to come on to using NOD SCID mice and also Deng's group in Sydney. As you will see in a moment both of these report limited infection in this model. There have been other studies including again from Yoken Denny's group using rats and immunosuppressed were getting no infection. He has, also, done a guinea pig study collaboratively with us and not been able to detect infection which is in contrast to the work of my colleague Danny Galbraith who has demonstrated infection of guinea pigs, and I want to come back to

that and see how I think that dichotomy of data might be resolved.

This is Deng's study and similar in sort of initial design to the study of Dan Salomon's group, involved transplantation on the kidney capsule of islet cells and these animals were followed over 3 to 41 weeks and tissues were harvested from various organs including the liver, the spleen and kidney itself and analyzed by PCR for PERV and also by PCR for mitochondrial cytochrome oxidase II. Of course mitochondria there are in multiple copies. So, it is possible to use this as a sensitive indicator of microchimerism.

Unfortunately these are standard PCR assays and I would like to comment later that in fact this is not the ideal way to do this study, but nevertheless they concluded from these data that there was microchimerism in 79 percent of the samples they took and based on the ratio of having PERV positive but no cytochrome oxidase II-positive samples that there was evidence of infection in two samples and these were concluded to be PERV-A.

It was a bit of a curious story because one of the viruses appeared to be defective. So, it is an interesting situation, but in none of these mice that were positive, neither of these mice which were positive was there evidence of PERV transcription.

Now, there is a theoretical slight problem when you use mice or other animals that contain their own endogenous virus and it is shown here, and that is that mice produce their own retrovirus and they don't often produce this virus but we have to take it into account. They produce virus which is called dual polytropic which can infect both human and mouse cells and indeed pig cells. So, this virus could come out of a mouse cell, could infect a pig cell, rescue the genome of the pig cell and it coats round the genome of the pig cell. That is called serotyping and it can then carry that virus from the pig cell into other pig cells and indeed into mouse cells. So, this virus could be carried around by the mouse virus. So, that is always a caveat for these types of experiments which one just has to bear in mind.

Now, I want to turn very briefly to, I am not going to go into details of analysis but this particular technique is important in the conclusions derived both from the human studies that Dan will be talking about and also from Dan's own study on islet cell transplantation. Increasingly we now use techniques of PCR that are real-time quantitative PCR approaches. It is like a standard PCR with forward and reverse primers, and you have a probe in the system that contains reporter dye and a quencher dye. As this is extended during the PCR reaction the reporter dye is cleaved off and can become detected by the apparatus because it is free from the quencher and as you conduct the PCR over many cycles there comes a threshold cycle where the machine will detect that fluorescence above certain predetermined controls in the system and that is called a threshold cycle.

If you plot the threshold cycle against the starting copy number of the DNA in the system it is linear and to actually show this this is actually data from my colleagues in Glasgow, I am just showing with a PCR to porcine endogenous retrovirus and you can see that you get a high correlation. It has got a correlation of 0.964, effectively a wide range of being able to detect PERV here down to 10 copies in this particular reaction. Now, the advantage of that is that you can take this situation; this is again based on Dan's data. You can look at a porcine cell and you can get a ratio of the number of copies of PERV to the number of copies of mitochondrial repetitive sequences and you can work out a ratio and a standard deviation of that ratio. So, consequently when you go and look now and here are some mouse cells, and here is a pig cell that has been transplanted in you can pick up that pig cell using say the mitochondrial probe at around 1 pig cell in 10 to the 5 or even better.

Now, the problem arises how do you tell if those mouse cells have become infected. Well, in fact if the PERV to mitochondrial ratio increases that becomes a priori evidence for infection of those mouse cells, and in fact they were able to show that – I think I have got that wrong. It is 1 in 1000 cells, when 1 in 1000 cells become infected this can be picked up in the background by using this quantitative PCR and comparing the ratios. So, you can pick up 1 in 1000 infected cells in this system and this is the result of the experiment I have just been alluding to from Dan's group.

They were able to show that islet cells would express PERV proteins in transplanted mice by looking by fluorescence and also show that these produce virus *in vitro*. In fact, that RNA expression increasing up to 10-fold after transplantation implied that maybe part of the cytokine profile during transplantation may increase it. They also observed extensive microchimerism in many tissues not just in tissues outside of the transplantation area and again picked up I think it was two cases of infection in murine cells at sites where the microchimerism was present. Infection was not detected where microchimerism was absent and so, this again perhaps implies that really close cell-to-cell approximation is required for this infection.

Finally, I want to turn to a study that my colleague Danny Galbraith was involved in in Glasgow, and I am a little bit puzzled by this. We don't know quite what is going on. This was a study in which we used a subgroup B virus coming from 293 cells. We used high-titer viruses. I actually counted virions under the actual microscope and actually we had 10 to 6 virions in the system, and I should emphasize that the first, this was not an experiment to look at infectivity. It was actually just a tool to raise antibody against PERV. So, the first shot was given with Freund's complete adjuvant. It wasn't an infection study and of course there is no infectious virus. The second shot, however, given 28 days later contained infectious virus, and we can demonstrate that it was infectious and these guinea pigs developed antibody and quite reasonable antibody titers by Western and by ELISA.

When we looked in the spleens and Danny just had the idea he would go and look in the spleens of these animals and he looked at the spleens of eight of these animals and all eight had actually proviral DNA present and at quite high levels. He had, also, looked for 293 cell DNA. Remember this is virus gone in, not cells, but he wanted to exclude that this was due to passive uptake of DNA that might have been in the injection and he couldn't detect 293 DNA. So, it would imply that this probably was infection and similarly of course he had controls here which he looked at contemporarily and he couldn't find DNA.

Now, the puzzle about this is that Yoken Denner and I believe Caroline Wilson have looked and not been able to repeat these data. So, it may be an experimental artefact, but I don't think so. I think what might be going on, and this is hypothesis I should emphasize, not fact, what might be going on is the possibility that because we have got antibody in these animals that when we give the second shot we are actually getting enhancing antibody that binds the virus and is carrying that virus into reticular endothelial cells in the spleen. I should add that this is actually a known phenomena with other species. In fact, in the early days when vaccines were being developed against feline leukemia virus the early vaccines actually enhanced infection rather than reducing it through a mechanism that was thought to be very similar to this one. So, I think that may be the possible solution to this difference in the data.

In conclusion then we have got no evidence of PERV infection in primates *in vivo* although the cells can be shown to be permissive *in vivo*. Immunodeficient mice display low-level sporadic infection which may require cell-to-cell contact. There are some conflicting data in guinea pigs and just as a hypothesis only it may be that enhancing antibody may be responsible for the data of Danny Galbraith.

Now, finally very briefly I just want to turn to the final point, with a couple of slides just to show what

happens or what we are doing now in Glasgow. This is the work of my colleague in the university, Dr. Linda Skobie who has been mapping the proviral loci in pigs. You remember that there are about 50 copies in pigs and what she has been doing has been looking in libraries and then mapping exactly where they are in the library. What you can do is you can find that some of these are defective and once you pull these out you can, also, look at the flanking sequences to generate unique probes that identify that locus and you can then determine whether that locus is occupied in any given pig by a retrovirus or whether it is absent.

So, this allows you to look at the genetics of provirus inheritance in pigs, and this is a summary of an awful lot of work done by Linda. On this I should say that this work was, also, collaborative with the Novartis group in Cambridge. As you remember there are about 50 copies of PERV in the pig. If you then screen both with env and pol you end up with only about 11 copies. In other words most of those proviruses as Clive has pointed out are highly defective. They are just bits of junk. She then with colleagues in Novartis then did another elegant sort of follow on, and that was to look at protein truncation testing, that is to find out whether these full-length viruses, whether they are capable of making a complete full-length protein or not and when you do that this reduces it down to six copies. In fact, two of those copies are duplicates. So, actually there are only four copies of a fully functional provirus in this particular pig.

She has now developed flanking sequence probes to this which has enabled Linda to go in and look in many different pigs both in the same herd and in other herds to look at the zygosity, the variability of inheritance of these viruses and it is surprisingly variable, and essentially what I think this means as Clive has pointed out that it will be possible although we cannot formally say that, I think it will be possible to breed pigs that do not contain full-length proviruses by appropriate crossing experiments. Whilst that I think is technologically possible it may be simpler in fact, oh, sorry, this was just to show I should emphasize that this is a mini pig line. This is not Clive's, but it is another one just to show that there are many copies of C in many pigs, but subgroup C virus is missing from certain cell lines and from certain pigs.

As it happens in the pig I have just shown you with the four copies there is no C virus. There is no subgroup A virus of full-length probe either, confirming what Clive has pointed out that those four proviruses are all subgroup B and it concurs with much of the data that Clive has been developing. Should we not be able to breed them out or sorry, even if we cannot breed them out or even if we can it may be technologically simpler now to go to gene knockout technology. There are now three groups of course who have developed pigs by cloning by taking out an adult cell, putting that adult cell into an egg and then transferring that into a pregnant, pseudopregnant animal and getting piglets out. So, knockout technology has not been applied to this yet, but it is only I think a matter of time before that becomes feasible.

Thank you very much.

(Applause.)

DR. KASLOW: Excellent, thank you very much. We now have a little bit more open time for questions to either of the last two speakers and then we can move to a period of public comment before we take a break and then come back for the last two speakers. So, are there first questions for Dr. Patience or Dr. Onions that we didn't get a chance to address before?

DR. POTTS: Clive in our Chinese hamster ovary cell line that we used to produce our product we are

required to have extensive electron microscopy done to look for type A, type B, type C, type D retroviruses and have you done that with the pig cells or do you plan on doing that to have a really good diagnostic test done in a GLP setting? I think it might be worth it.

DR. PATIENCE: With respect to looking by EM we haven't looked by EM. The methodology that we used was a conserved PCR approach that identifies sequences of viruses which don't vary between different viruses and we published a couple of months ago in *J. Virol.* showing that the infectious viruses seem to be limited to this PERV A, B, C family and there are other PERV but they really are junk DNA.

DR. POTTS: Now, when I say, "A, B, C, D," I don't mean of the C-type family but of the A-type family, the B-type family.

DR. PATIENCE: Yes, that was what we published on; the viruses related to the B and the D type are present in the pig but they do not have an infectious capacity.

DR. POTTS: Oh, okay.

DR. ONIONS: We have actually looked by electron microscopy in both primary pig cells which are releasing type C viruses, and in fact some are not and we have not been able to observe, we never observed the type A particles or type B retrovirus.

DR. VANDERPOOL: A two-part question. The first question is both of you commented which is quite exciting news that you can see the development of PERV-free pigs in terms of human infectivity. Do you have a time line on that breeding, on those breeding techniques?

DR. PATIENCE: May I start that? I think a lot of those animals which you are looking at up there at least were failing in transmission assays and they are the exact animals that we are going to be looking at at the genetic level to see if they are lacking these viruses which have been identified. Again, interestingly the PERV-A which David failed to find an infectious one is the only virus or beta recombinant that we find in any of the transmission assays. So, it is looking very encouraging.

DR. VANDERPOOL: Okay, the second part of my question is could one conclude that once these developments are made and assuming as we all assume that these pigs would be developed and grown within pathogen-free environments is it fair to say that what remains is to overcome the immunological barriers and to know more about physiological function and then we are ready to move toward organ xenotransplants?

DR. ONIONS: I feel more optimistic than I have felt for some time to be honest, cautiously optimistic. I think there are some still formidable problems though, not to comment on the immunology but on the virus side. We will probably hear from Barbara about the importance of certain viruses like circoviruses and so on, and some viruses which have crossed the placenta which Clive mentioned which are difficult to get rid of. Nevertheless those are solvable problems. I think they are solvable. So, yes, I am cautiously optimistic that the zoonotic issues can be solved.

DR. PATIENCE: I would just add I think it was really the mind set change which I think is becoming more appropriate where when PERV were first described people thought this was not a problem that could be overcome, but it really does look like it is a feasible issue and as David says we then move on to the rest as it were.

DR. VANDERPOOL: The other second part of my question actually I had three parts I guess is that it is really quite interesting when PERV was first seen as infecting human cells *in vivo* there was quite a negative response to kind of a disappointing response to the possibility of organ xenotransplants, porcine transplants and yet the work we are getting from the two of you is that even though we had this worry in 1997 and 1998 in those times that a lot has happened since then and the worry over PERV has very significantly decreased. Why is it then and maybe this is a question of differing cultures more than a question of available scientific evidence, but why is it then that the UK advisory committee was so almost pessimistic about moving forward with vigor? I experienced the time when both the Nuffield Council report on xenotransplantation and the Institute of Medicine report were presented to the public at the same time, and I was one of the spokesman and I think David perhaps was one. Were you there? I am not sure whether either of you were there, but I began to sense that this was really a cultural difference between a more conservative approach in the UK and more sort of "go for it" approach in the US, but regardless of whether that observation has any truth to it how would you account for the conservative statements made by the UK advisory group?

DR. ONIONS: I think it really has to be seen in a European and not just a UK context. If you look at the actual guidelines that exist now for xenotransplantation they are really very negative. They actually say that we cannot go ahead until there is international agreement on things like patient monitoring and so on and so forth. I was actually at a UNESCO meeting last week where this issue was discussed, the ethics of xenotransplantation and there is I think a real reservation about the whole procedure primarily because Europe has been scarred by the BSE epidemic. It has had a real psychological effect and it shouldn't be underestimated although fortunately we are still, you know, only just over 100 people have died from this disease. There is this fear of this becoming a major epidemic and therefore there is a consequent immediate gut reaction by many people and even informed people that this is just an unacceptable risk, and if you like that same attitude, of course, also, holds in Europe to GM foods, and there is a different cultural reflection on these two issues, and I don't think that will be changed very easily, I am afraid.

DR. VANDERPOOL: So, part of the challenge to this and other committees would be to do some education of the public about where we are because it seems to me that there have been these cultural factors that have led to somewhat of a withdrawal of enthusiasm in xenotransplanted organs.

DR. PATIENCE: I utterly agree. I think it is critical that the flow of information comes from the research bench through the Committee and to the general public.

DR. SALOMON: I think as a follow-up, I think as a Committee we ought to realize one of the lessons we have learned here is that an intelligent public discussion led to identification of porcine endogenous retrovirus as a risk and that set into motion over a relatively short period of time a remarkable series of scientific investigations so that really 3 or 4 years later we are very close to understanding a lot more at least. There is a lot more to still learn, but we have learned a lot about the virology of the virus, about its infectivity, about its range. That is a pretty solid recommendation, I think to good science applied to a public health issue that then would allow you to make informed decisions at a regulatory level.

DR. SYKES: We heard some very encouraging studies of intentional PERV injection into immunosuppressed non-human primates. I am just wondering in view of what we have heard about anti-gal being protective against PERV infection whether there are plans or whether such studies have been done with animals in which anti-gal antibody has been immunoabsorbed or has been absorbed?

DR. ONIONS: Clive may want to comment because he has done some work in this area. In those animals that I mentioned at the end it probably should not have been a major issue with the anti-gal

because those viruses were grown in human cells. So, they should not have been carrying the epitope. So, in fact, that is why it was done. The idea there was to maximize the probability of getting infection but I think the comment you made is very pertinent and that would be a useful study to perform as well.

DR. SYKES: That is even more encouraging that those have been passaged already through human cells.

DR. PATIENCE: What we need to take it a level further is to try to mimic what the knockout pig would look like. So, I think I utterly agree with David's comments that those viruses were hotted up and from human cells. We are putting another layer of security on in addition to that type of infection study and looking at knockouts.

DR. KASLOW: Any other specific questions or comments on these two presentations? If not, I think we will, excuse me, yes?

Agenda Item: Public Comment

DR. HART: Hi, I am Aretha Hart from FDA. I just was intrigued by your genetic loci study. It sort of reminds me of the analysis in the mouse system for endogenous retrovirus and in that system as you know it was very useful to investigate endogenous retroviruses and the regulation of expression using a variety of viral inducers, and I was wondering if you have investigated along those lines.

DR. PATIENCE: Certainly in the tissue culture assays we have not only addressed various inducers; we have, also, done various cell sources to try to absolutely ensure that we activate all loci and we find that the PBMC is by far the best source of virus to be used. So, we have tried calcium ionophors. We have tried mitogens with and without esters. We have done quite a range.

DR. HART: I was just thinking of the typical retrovirus inducers like IDU or azocytidine which are demethylating agents especially in terms of analysis of the miniature swine model to ensure that there are no endogenous infectious viral loci.

DR. PATIENCE: That is exactly the type of study that we have done, not those particular demethylating agents but demethylating agents have been put in.

DR. ONIONS: It is possible since we are commenting on Lynn Scarbisari in the studies she did of isolating these clones she is currently looking at whether these can be transfected into cells and produce infectious virus. Interestingly despite these experiments being done multiple times in multiple ways so far we haven't actually got an infectious virus out of those four clones. So, it is possible that there is maybe something the LTRs that is preventing transcription in human cells or some other factor. So, where these contain full-length function proteins so far these have not been transcriptioning out to human cells which again goes back to Clive's point and adds yet another level suggesting that in fact in most pigs actually getting a hot infectious virus is actually very difficult.

DR. MANG: We, also, did some research work for the PERVs and I want to give some general comments, that is besides these PERVs A, B and C, last year we also identified one full-length sequence. So, we call it PERV E. While we call it PERV E, that is we analyzed the whole sequence of this new PERV and then we compare it to the known human endogenous retroviruses and we found it is very homologous to the human endogenous retrovirus E family. So, actually we give this PERV E and then actually after the identification of this new virus we tried to find out the PERV E transcription from the

different tissues and in the first one we look at the pig placenta tissue but we couldn't find it and then later on actually in this year in the March issue of the Journal of Virology Clive published that paper about the gamma virus and the beta virus and then we, also, did some analysis. We found out that the virus we identified which we called PERV E is exactly the same virus which you described for the gamma 2 and in that paper you described, you confined the transcription in the kidney tissue of the pig. So, this is what I mean, just like what you mentioned during the presentation. The recombination between the different retroviruses is also quite a general issue. So, I am wondering how do you consider that if that is PERV. One of the PERVs also has been homologous to the certain kind of HERV. Then if the possibility between these two different kinds of endogenous retroviruses they can recombine with each other and then generate a new species. I don't know because this is, also, my major personal question. So, this is what I want to present here.

DR. PATIENCE: I think I will sort of probably make reference to David's presentation where that theoretical possibility is recognized I think when we are dealing with levels of safety though stemming the flow of any viruses at the genetic level where the pig starts is ultimately going to be the best place to control any risk. It will never be zero but neither will very, very many things.

DR. KASLOW: All right, thank you very much. We are going to now move to a period of 15 minutes for public comments, if there are any. Is there anyone who wants to make such a comment? All the questions are answered. Okay.

Dr. Laderoute from Canada had expressed some interest in making a comment. Please if you care to do that?

DR. LADEROUTE: My name is Mary Laderoute, and I am from Health Canada, the Center for Infectious Disease Prevention and Control, and I have actually worked on the regulatory side of xenotransplantation in Canada, and I know some of the people here because they were at our forum in 1997, but I have switched over to the surveillance side.

So, I am, also, from the blood-borne pathogen division and we have a couple of recommendations for the Committee, the SAC Committee to consider. However, we are infectious disease experts and/or immunologists. We have no advice to give you on the ethical, legal or cost/benefit analysis. So, you will have to do that yourselves, which is probably harder than the infectious disease risk.

So, I tried to make it as simple as possible for today because we have quite a broad audience here, and what I have come up with is approximately 11 general areas of concern.

Now, we are not saying that this is the way you should go. We are just saying, "Look at these issues," but before I go into these 11 I do have to stress that you are going to need a lot of resources for the research aspects and I think from what we have seen at the forum and what we have seen at other discussions internationally is you really need a national body such as yourselves to ensure that there are enough resources available, for example, in the case of an emergency breakout for the development of appropriate screening tools, etc., So, I put the plug in there for you, Louisa.

So, it is resource intensive. Don't get me wrong and that is a big consideration, but let us move on to the 11 issues and before I do I just wanted to -- I was told I had 5 minutes, but really this presentation takes 10 and I really wanted half an hour. So, what it is is a year ago I had a meeting, a workshop on xenotransplantation surveillance issues, and I am happy to say after a year and one-half we finally have something on paper. It is coming out on the Web site I think next week, but I have asked for this

workshop report to be distributed to all the panel members. In addition to that I made a printout at the front of each of the workshop copies, I made a printout going into a little more detail about the 11 points because I won't have time to really go into much detail today.

Okay, so, next slide, please? Okay, these are basically just broad issues and this relates to reduction of infectious disease risks. It doesn't mean you will make the risk zero, and it doesn't mean that this is a guaranteed way to actually reduce risks. Like I said, it is up to this Committee to decide all the legal, ethical and cost/benefits and the efficacy of any given recommendation, but from the workshop that I held one overpowering thing that came out of that was I really believe you need to expand the definition of reportable adverse events for xenotransplantation clinical trials. So, in Canada we have developed what is called proposed draft standards for xenotransplantation, and we actually defined what the adverse events should be that should be immediately reported. So, for example, cancer is there. Graft failure is there, but you are not going to see that defined for other clinical trials because it is usually irrelevant, but also what is very important I think because of the next issue is a death should be reported immediately irrespective of the cause.

Now, in many countries including Canada to some extent deaths are not reported in clinical trials because the physician feels it is not related to what is being tested, and the reason why it needs to be reported in xenotransplantation I feel is for the next issue and that is how are you going to know if those PERVs are not in the kidney of the human or in the bone marrow unless you test. Testing peripheral blood is great, but it is not the end all, be all. You don't have two or three hundred tissues represented in the blood. So, the second point relates to potentially mandatory consent for investigation of the agents in post mortem samples. It is very important that you get those post mortem samples to look at for the various infectious agents.

Next slide? This is an ethical question. So, you will have to get your ethicists on board on this issue, but there is an easy way to prevent vertical transmission, transmission from the mother to the embryo or to the new baby and that is to make sure that the women who are in the early trials cannot have babies or men are not fathering children because they are physically unable. You might, also, consider because of what we heard today and about the issues just raised about recombination, you might not want to be transplanting into HIV patients, etc., or other people who might be immunosuppressed.

You never hear too much anymore although we hear about mad cow disease a lot we don't hear too much in xeno about minimizing TSE risks. Now, I know that the pig is naturally resistant to TSEs but it has also been shown to transmit to a third species. So, I think here what we are suggesting is speak to the TSE advisory group, get a subcommittee going who can look at how to minimize these risks. So, for example, a simple easy intervention is cheap, relatively cheap. It is to make sure that all your surgical instruments are disposable when they are used for xenotransplantation. So, there is a whole list of issues that you can look at, but we are saying, "Please look at them."

Next slide? We heard a little bit about, actually we always hear a lot about the endogenous PERV but also endemic viruses. So, there is a whole slew of viruses out there. A lot of the herpesviruses are encoded in the endemic, but I think and we think in Canada that there is an international body to actually do testing, and the reason for that is well, we had this crisis, and I don't know if you are aware of it, but this was the E. coli breakout in Western Ontario and it is because they used private labs and the people weren't actually doing the testing when they were supposed to be. They said, "Yes, we were doing it," but they actually weren't. So, I think it is better for public health that you actually do the testing yourself which also means that you will have to archive samples yourself.

Spousal informed consent is a touchy issue but again you have to get your ethicists out, but really if there is sexual transmission and we heard this morning how important it is, if there is sexual transmission to actually test. So, manage your spousal informed consent to do that and the national registry and archives kind of go hand in hand.

Next slide, please? I am trying to rush through this so not to take up too much time. Oh, yes, dedicated inspection teams. I am sure most of you have not heard about our semen programs in Canada. Yes, we had to import from the States. So, we hope your stuff is good down here, but the problem with the semen regs was that no one ever inspected these places that were storing, sampling, testing semen and when they went and did their inspections I think they shut down and actually put out of business 18 companies, out of business, gone because they never did any initial inspections. So, while that will delay how long it takes to actually get an approval or authorization of an IND I think it is important to do your inspections at the beginning. So, we are talking the animal sites, the transplantation sites, etc., but you need a dedicated team.

Public disclosure. Actually there was, I think it was January of this year there was a move for public disclosure the first of its kind for gene therapy and for xenotransplantation clinical trials here in the United States, and we welcome that.

Next slide, please? I did go to that Paris meeting about international cooperation and standardization and we need internationally standardized definitions to work with as well as monitoring and perhaps because I am from the Centers for Infectious Disease Prevention and Control here is my favorite one. In addition to an emergency plan if you do have an outbreak or a major outbreak my personal bias is that you, also, look at non-toxic virals, for example herbal medicines. There are lots of herbal medicines that may actually have or are suspected of having very good antiviral activity and so I would like to see some of those evaluated and research monies put into that so that if there is an outbreak you would have at least something to work with and there are more details in that handout and I am sure if anyone wants one the Chair will provide it at a later date. I have an electronic version for him as well.

Thank you.

DR. KASLOW: Thank you very much.

Dr. Vanderpool?

DR. VANDERPOOL: Thank you very much. We appreciate all of these suggestions, and we will scrutinize your document and we appreciate your 11 points. Several of these issues have been and certainly will be addressed by this Committee, and you have brought to our attention a number of issues that we haven't discussed such as third-party consent, but continue to reflect on these issues and continue to give us your wisdom on how we should proceed because we do appreciate the thoughtful input of our northern neighbor, Canada.

DR. KASLOW; Are there other public comments, comments from the audience about any of the issues or aspects of the discussions we have had so far today?

All right, if not, yes, Dr. Onions?

DR. ONIONS: Could I make just a very brief response to the last presentation? I think there is an error of fact that should be corrected. Pigs are not resistant to TSEs. So far BSE has not been transmitted by

the oral route to pigs but if you inject it intracerebrally pigs will quite competently develop a TSE. The reason TSEs are not such a major issue in xenotransplantation is that they are not a public health issue. They are a risk to the individual patient and probably given the fact that pigs are going to be used at an extremely young age the probability of having a spontaneous TSE in a pig is somewhere in the region of probably 1 in greater than 10 to the 10. So, it isn't a public health issue. It is a very, very low risk issue to the patient.

I have one second very quick comment and that is that in terms of the risk to vertical transmission in all of those systems where we know there has been re-endogenization, the work for instance of Jonathan Story and others it is known that that is associated with high-titer viremias and that would require a patient to be having a high titer per viremia for endogenization to the germ line, a situation that we would certainly pick up.

DR. KASLOW: All right, thank you. We will take a 10-minute break I think and resume at approximately three-forty.

(Brief recess.)

DR. KASLOW: Okay, the first of the last two presentations will be by Daniel Salomon from Scripps Research presenting Retrospective Studies in Patients to Detect PERV Transmission and Pathogenicity.

Agenda Item: Retrospective Studies in Patients to Detect PERV Transmission and Pathogenicity.

DR. SALOMON: Thank you very much. This is a triumph in technology to get this to work from a Mac to a PC environment. I always breathe in relief when it does work. So, I am going to pick up with a brief discussion of the human clinical trials in xenotransplantation that I think obviously the background was said beautifully by Drs. Onions and Patience.

So, a series of preceding papers in human clinical trial experiences became summarized and if you will republished here in a better forum in this key paper in 1999 by Kaz Paradis et al, and this included now something that I think is very interesting. Historically this was actually a study done with Novartis, Imutran funding and I think it shows the kind of cooperation that large pharma and biotechnology and academia can make in an area in which public health issues are important and I don't think that is a minor thing in the history of xeno. I think this is a very, very positive paper even before you look at the results.

Now, the purpose of this whole thing was to essentially cast a worldwide net and ask anyone and everyone who had had any experience with a patient under any circumstances at all in which pig tissue was transplanted or exposed through extracorporeal circulation to a patient that we wanted to know about it, and it had some interesting results.

First of all there were a total of 160 patients identified worldwide. Extracorporeal splenic perfusion was a gigantic surprise. So, a group of physicians in St. Petersburg, Russia had decided that over about a 4-or-5-year period that patients with severe burns, sepsis and/or other acute stress situations needed a boost to their immune system, albeit temporary and the mechanism for doing that was to perfuse them for several hours across pig spleens and they were blissfully unaware of any possible problem with doing that and were extremely cooperative in sharing with us the results of the 100 of their patients. We are not really certain how many patients they actually did and more frustrating to me is about a year ago I tried to follow up on this to determine whether they were still doing it, and all I could track down was that they had left St. Petersburg but that someone at St. Petersburg thought they were still doing it somewhere.

I find this reassuring, but I think you need to put this into the context of what is happening out there in the real world, and there were the things that we knew about, of course, but they get a lot of cooperation here from these groups. So, this is the bio-artificial liver where pig cells are loaded into hollow fiber kidneys across a small porous membrane, basically little capillary fibers and then you counterperfuse patient blood over them. There were 28 of those patients.

There were a number of pig skin transplants that were done in acute burn patients. Remember that none of these patients are immunosuppressed at least in conventional terms albeit these are patients with acute liver failure and that is not exactly unimmunosuppressed. In other words, there is certainly a whole host of immune dysfunctions in that setting. These patients here had a whole number of diseases but acute burns, for example, in the case of the skin and some of these patients with sepsis is, also, not normal immune system. So, these are just things to keep in mind.

Pancreatic islets, this was from two different groups. The majority of the patients came from Carl Growth in Sweden and again this was published separately. This was published separately. What did they find? They used the most sophisticated techniques that were available. They used the Taqman quantitative PCR that Dr. Onions described to you. They did Western immunoblots for antibody which is a very good, very sensitive way of looking for retroviral infection exposure and they found no evidence of PERV in any sample. This involved CDC investigators. This involved industry investigators. David Onions was one of the investigators with his company Q-One Biotech. There were again people from Imutran, Novartis, etc., and interestingly there was persistent microchimerism.

In other words, pig cells were detected in the peripheral blood of 23 patients of 160 some 8-1/2 years after exposure. So, I think when this was published this is probably the most, not probably, this is the most extensive experience published on human exposures to xeno tissue to pig tissue and at the end what I am going to do is just review these kind of briefly with some editorial comments and then my fifth slide is essentially going through sort of like why didn't this happen, and we will go back to this detail.

This is a paper that just came out from Levy et al last year in which they did allotransplantation after extracorporeal hepatic support with a transgenic, now a dual transgenic CD55 and CD59. So, essentially these are these complement regulatory proteins that were discovered on the surface of endothelial cells. Essentially they are involved we believe normally in deactivating complement that normally would be activated at the surfaces. It is a kind of protection or counter-regulatory mechanism to reduce endothelial injury in various situations, and the idea was if you over express these human complement regulatory proteins on the endothelial surfaces of pigs through transgenic engineering you would significantly reduce the impact of natural antibodies activating complement locally and I think the rest everybody is fairly familiar with.

So, in this case this is extracorporeal circulation but not across a hollow fiber barrier but actually liver to connection to an actual blood system. So, this is a very close exposure to the patient. Two patients only are involved in this study. They were bridged with transgenic livers for 6-1/2 and 10 hours respectively and then they were transplanted with human livers and at 5 to 18 months later there was again no evidence of PERV infection by a series of assays. So, at least there was immunosuppression there but the immunosuppression was done post-exposure. There is no, interestingly they don't mention any evidence for microchimerism of pig cells in that study which is a little bit surprising in the sense that liver transplantation in humans has been one of the organ transplants that has been the best associated with chimerism of donor cells in peripheral compartments.

This next one came from Dinsmore et al, and this was mentioned by Clive. So, there was no evidence for

infection of human cells with porcine endogenous retrovirus after exposure to porcine fetal neuronal cells. So, this was the 10 patients with eight controls that was just recently reported of fetal pig mesencephalon cells into patients with therapy-resistant or therapy-unresponsive Parkinson's disease and again they found no evidence of PERV transmission.

Now, here there are two interesting things. One point here is they found no evidence of PERV transmission *in vitro* from the pig neuronal cells that were used to do the transplant. So, what that is beginning to set up is that not all pig tissues, at least not under all conditions are shedding infectible PERV virus and that raises some interesting questions then as to what sorts of things we need to consider in terms of looking at for example the safety of different kinds of cell transplants as well as the different kinds of *in vitro* culture protocols that are used to test these. So, one has to be cognizant of weaknesses on both side of this. There was no evidence of PERV for viral DNA but remember these were tested in the peripheral blood lymphocytes from the 24 patients transplanted under several different conditions, at least 10 of which were immunosuppressed.

The problem here of course is to No. 1 at least I have never been successful in infecting human peripheral blood lymphocytes activated or otherwise with porcine endogenous retrovirus and that has been the experience of Carolyn Wilson and others. Now, there are lots of good people in this room and if someone wants to raise a hand later and say that they have managed to do it, I will stand corrected, but the idea that a peripheral blood lymphocyte is a very good target a priori is unclear to me. Secondly, certainly intracranial transplantation and then looking at peripheral blood lymphocytes has its own barrier to consider.

This is an interesting one. I think if you had asked me to do it I would have made some kind of glib comment about it, but after thinking about it, I thought, you know this is important and I realized how many people have called me and asked me about whether or not these pig heart valves could be causing infection and of course I would roll my eyes and say, "That is ridiculous," but this is really the first solid evidence scientifically done, and I realized it was very appropriate. So, a group in Germany did this study in which they took glutaraldehyde fixed pig heart valves. They took blood samples from 18 patients that received these basically fixed heart valves that had had the valves from 6 months to 3 years and they were negative for PERV infection. They did fresh pig valves and they were positive as one would expect and then they put them in glutaraldehyde and checked them every day for several days and again you know this is sort of trivial but very important and so I am taking it very seriously.

This was mentioned, also, by Clive briefly. I think this is an interesting one to think about though. These are two papers, one from Walid Heneine and his colleagues at the CDC and the second one from Carolyn Wilson's group at the FDA and they both looked at a commercial preparation of plasma from pigs being used to treat hemophiliacs and this had been going on for years. By the way the heart valves also have been used for a decade or more and both papers demonstrate that PERV particles by both RT, reverse transcriptase and PCR reactions were present in these pig plasma preparations being used in patients particularly in Europe and France is where these lots came though they were produced in the United States and I remember when we first heard about this at the Cold Spring Harbor symposium there had been no further work, and this was an amazing result. I mean we were all like, "Oh my God, is there going to turn out to be an infection already in France in human patients?"

It turned out sort of interesting. Even though there was our reverse transcriptase activity here which suggests the presence of a potentially infectious viral particle, albeit the virologists will quickly confirm that RT activity is not the same as you can have a defective viral particle with RT activity, but they evaluated 88 patients that had been exposed to this preparation for PERV antibodies. They were all

negative and the conclusion was that PERV risk in the setting was low. Now, at that point one of the ways you could spin this data is to say, "Well, you see even if you have infectious PERV it is not infectious to humans, at least not immunosuppressed humans and derived from pig cells."

However, what was interesting was in the second paper it was found that these PERV particles were not infectious in *in vitro* culture. So, at least within the sensitivity of the *in vitro* cultures these weren't infectious virions, more than likely because they represent endogenous pig PERV being produced and it is mainly the type C which does not have amphotropic(?) envelope and is not infectious for human cells though interestingly enough neither of them finished with stating that though I have heard that informally from Carolyn and Walid.

So, this is my last slide.

Let us just kind of go through this carefully. The question is just an obvious one. It seems like I need to end with this and that is we started off 5 years ago with clear representation from Robin Weiss and Clive and David Onions and Carolyn and myself and others that PERV could infect human cells in a series of publications and that raised everybody's limit and so the question one has to ask in trying to finish here and put it into some context then why isn't this a no-brainer then. I mean PERV gets into human cells and why didn't PERV cause any clinical infection, and we need to think about a couple of different reasons. There is not one.

Let us take this one, cell lines versus primary cells. The majority of these studies have been done in cell lines which are adapted to culture, grown in plastic on fetal calf serum, you know, no complement. This is not a real situation, okay? And in fact the majority of these studies have been done with a human embryonic kidney epithelial cell line called 293 which we all love because it is just very easy to infect. It expresses beautifully and it is great for doing the kinds of basic scientific research on different sorts of viruses and viral vectors for gene therapy. So, we all like it, but a lot of this stuff was done with that.

Now, there have been some primary cell lines infected. For example we and others have infected human umbilical endothelial venous cells, UVEX(?) so it is not that no primary cell lines have been done, but nobody has done a really systematic approach where they have looked at primary kidney epithelial, primary hepatic epithelial, primary seminal vesicles, you know just different sorts of epithelial and endothelial and fibroblast cell lines in the human.

Okay, exposure. There is a bunch of different issues to briefly discuss with exposure. First of all a lot of these exposures were very short term. Some were perfusions that were hours long. Others, there was a question of how long anything survived. I mean we will get to the microchimerism question in a minute but many of these studies a lot of these non-human primate experiment studies for example had no evidence whatsoever that the cells that were injected survived more than 38.5 milliseconds after they injected them.

So, I mean if you are going to have a decent study you have got to deal with the idea of how long these cells are alive and how long they are producing virus and how much virus they are producing locally and where they went when they produced the virus, etc., and some of these were in isolation procedures like the hepatocytes loaded into the hollow fiber capillary tubes or encapsulated islets in some of the pig islet experiments in which there are other reasons to suspect that the efficiency of viral transfer is either inhibited completely or significantly reduced.

Pig producer cell type, I made the comment that the data at least the studies done by Dinsmore et al on

neuronal cells showed that pig neuronal cells didn't seem to produce PERV and David Onions and Clive Patience both pointed out that we did studies that showed that islet cells, for example, and Carolyn Wilson did peripheral blood lymphocytes and others have shown that other pig cells do produce infectious PERV so that it is important to think about the type of producer cell type. There may be some cell types that don't produce PERV. That would be great to know for safety in terms of demonstrating the need or the basis for going on to a clinical trial let us say of neuronal cells.

Viral dose. You know, viral dose is a critical thing to think about. You put in a few cells and you don't see infection. That doesn't mean the same as putting in cells that survive for a year and constantly are producing virus that can adapt, etc., and there are all kinds of issues about viral dose. I won't spend any more time on that.

Efficiency of cell-cell and cell-free transmission, we don't know enough about the biology of PERV yet, but our data in our mouse model suggested that only in the animals in which there were pig cells in the compartments did we find infection in those compartments which by the way were 8 out of 28 animals, just shy of one-third of the animals that we looked at had evidence of infection in the mouse tissues but not in compartments in which there wasn't chimerism for pig cells suggesting that cell-cell transmission may be an important mode.

If so, a number of these different sort of interactions don't afford cell-cell interactions and only cell-free transmission. So, there may be a whole efficiency issue that we need to think about in modeling how these things work, and there may, also, be a safety issue. For example, we may have a more reasonable safety view of encapsulated islet studies or extracorporeal circulation on hollow fiber kidneys than we would, for example, in a straight tissue transplant.

Activation stayed at the target cells. Retroviruses usually like to have activated target cells and so depending on where you are transplanting, depending on how much immunosuppression you give because immunosuppression does a lot of things, but one of the things it does is block cytokine signals to enhance activation and if you block cell activation locally or distally one might significantly reduce target cell susceptibility to retroviral infection and so different models and different uses of immunosuppression ironically, what I am suggesting is it is possible under certain circumstances immunosuppression would protect you from further infection that affects the activation of target cells. Microchimerism? Well, this is an interesting area. I mean they detected DNA by very sensitive PCR methods for pig in patients 8-1/2 years post-transplantation and that would suggest that there are still pig cells around. If that is true, where those pig cells are and what kind of pig cells are there is less clear.

Moreover if the pig cells that are there, let us say macrophages or dendritic cells which is usually the cell type that is considered to be the most common in terms of being the microchimeric cell, albeit it this is a controversial area and Megan may take me up on that later, but the fact is that until we know what cells are chimeric or cell is chimeric and then know whether that cell is producing PERV the significance of chimerism as a priori evidence that there must have been an exposure to PERV and the PERV wasn't infectious is not a correct leap of reasoning.

I think we made the points about immunosuppression. That has been a nice theme from both Dr. Patience and Dr. Onions, and I will leave it at that. It is pretty obvious. They have, also, really very much made the point of alpha 1-3 Gal expression.

So, clearly any sort of PERV that came from a primary pig cell budded from a pig cell surface carries the alpha 1-3 Gal residues, is going to be neutralized by human natural antibodies, but the fact is that

successful xenotransplantation implies by its very nature a successful avoidance strategy for this sort of natural antibody mediated injury. So, either you take away the antibodies or you tolerize with the antibodies or you remove them by immunoadsorption or you remove the sugars by transgenic engineering or whatever you end up doing, maybe the complement proteins are an exception but almost everything that you would end up doing in a successful xenotransplant would imply that you have obviated this powerful anti-gal effect.

Monitoring strategies just the obvious, right? Retrospective, all these studies were retrospective. There were no prospective studies in human patients. So, a lot of these patients you are just getting serum and blood. You don't know what happened really early. You were very happy that we got the kind of cooperation from these different groups that allowed you know from the Soviet Union. These guys were great in terms of providing plasma but they were random. We found this patient and it was 3 years ago, that kind of thing. PVL versus target tissue biopsies, as far as I know there is really no evidence for target tissue biopsy data. It is all done with PVL or with serum for antibodies.

So, my last question is is this snake dead or just sleeping? Right now the weight of the clinical data can be taken a couple of different ways. It clearly gives you some reassurance. I think if this were HIV even with all the caveats that I gave you in the interpretation of this data if this was an HIV-like virus I think we would have seen infection now. I think we have to take some comfort in this human work. It is of value. It is definitely of value.

What is happening though biologically is we are going from a virus that is an endogenous retrovirus. It is the remnant of an ancient plague. The potency of the virus that plagued the pig's ancestors is far from clear to us and finally, after generations of vertical transmission we are able to activate this virus in certain settings and so we are not looking at the true potential of this retrovirus. So, one of the questions one has to ask is how much respect should we have for just what a retrovirus can do unleashed into a new ecological niche as Dr. Patience pointed out to us?

Is it just making a couple of small changes like Dr. Mahy pointed out that would then give you a substantially more powerful virus so that a lot of these glib statements that some of us have made, and I have heard myself say that this is a wimpy virus and I have stopped doing that for this reason, you know or maybe it is a wimpy virus. I mean maybe there are just whole sections of the LTRs which drive the virus's biological function that are really just so neutralized and so poor and there would be so many gigantic amounts of mutation to cause the kind of, to resurrect a killer virus out of this that maybe there is nothing to worry about or maybe it is just a couple of changes here and there and until we really understand the biology of this virus I think we should be a little careful and treat it as a sleeping snake not necessarily a dead one.

Thank you.

(Applause.)

DR. KASLOW: Are there questions for Dr. Salomon?

DR. LUBINIECKI: I had a question. In the experiment with the high HC, the porcine factor VIII was that material subjected to inactivation by solvent detergent or heating in a lyophilized state or any of those methods?

DR. SALOMON: Not that I know of, no, because I think that would have destroyed the factor, but in

the discussion of the Takaperman-Wilson paper they mentioned that there might have been, you know, there was definitely a pooling and a purification of it to a modest extent, and they acknowledged that that might, also, have inactivated the virus or attenuated it, perhaps.

DR. KASLOW: Dan, I had a question. Was most of the material taken from the St. Petersburg group which was obviously the largest of the groups, what is the provenance of that material? Do we know anything about it at all? What documentation was there about when it was taken, how it was taken and so on?

DR. SALOMON: I wasn't an author of that, and I am going to defer that question over to Clive and David who might know better than me.

DR. ONIONS: I cannot comment because this was really Kazie's study, but one of the concerns we had was when there were rebleeds we were worried whether these were the same patients. I mean this sounds slightly cynical but we were concerned that in fact these were the same patients coming back. In fact, DNA typing of the fingerprinting was done on these, and they were in fact the correct patients. So, this was, also, supervised. You know, we were concerned about those variations that I think you were alluding to, but this was, also, supervised by Novartis who have obviously very good representatives on the ground, and we have no doubts thinking about the veracity of the collection. I think it was really well controlled and I have to say that as far as I understand the Russian authorities were very, very cooperative.

DR. SALOMON: Yes, I was on the advisory board as this study was developed and then pursued. So, I had an opportunity to, also, say, a couple of times you heard me saying, "We," I guess. I apologize. I didn't mean that I was on the study, but I definitely was involved in it, and it was wonderful actually how cooperative they were, and I see that as a great positive. I think they were completely innocent in the sense that they just never thought about it, and I think we need to think about it because there are lots of people out there who are completely innocent, and that is fine, but that is a part of the objective of a group like this.

DR. VANDERPOOL: Dan, I love your talks, and I love your analogies, but I am going to take you on on the final question, is this a sleeping or a dead snake. I used to live in West Texas, and I never did like a sleeping rattlesnake, and so that scares me when you say that it could just be sleeping. I think at least this snake has been run over by a few million years of lawn mowing.

(Laughter.)

DR. VANDERPOOL: And it may still have some life, but it is going to need a bit of resurrection to come to real life, it seems to me, and now do you disagree and do Clive and David disagree? I mean it seems to me this snake has had some serious damage done to it, and it is not just one that is lying there ready to rise up and bite anytime soon.

DR. SALOMON: I think what I said specifically was exactly that, that the original virus was thousands of years ago and presumably became an endogenous retrovirus, not an exogenous retrovirus because it devastated the ancestor of the pig and then that small group that survived managed to put it endogenously which provides then endogenous resistance, right? So, I agree with you. I mean that is your analogy to the lawn mower. I just remember, if you can tell a story then I can, when I was hunting in Florida we would go into the swamps, and one of the good old boys took me aside right at the beginning and said, "The only good snake is a dead snake, and if you kill them on the way in you don't have to worry about

them on the way out." So, I like the idea of Clive and David that probably the best strategy here would be developing pigs that didn't have PERV, but anyway.

DR. KASLOW: I still have to wonder whether or not you are looking at an old rotary blade lawn mower when in fact what we ought to be looking at now is a weed whacker, that is we are going to introduce this virus into a totally new environment, one that it hadn't seen during all those thousands of years of pig exposure, and probably a lot of whatever human exposure there was which was pretty casual by different routes with less immunosuppression and so on. So, while I am cautiously optimistic, I don't think we can presume that snake dead yet.

DR. SALOMON: And that is why I have taken a pledge not to say that it is a wimpy virus anymore because I agree with that.

DR. ONIONS: I think this Committee in the past has debated many times about risk and obviously risk is the probability of an event and a consequence, and I think when we are talking about clinical trials, the current data I think we have that is being developed suggests that the kinds of clinical trials that have gone on in the past and may continue are probably very safe procedures. The risk of PERV is minimal. I think the problem arises as we have always discussed is what happens when this becomes routine procedure, and we are hopefully treating tens of thousands of patients, and there it seems to me that the transition from going from our current position to going to a PERV-negative animal would not only be desirable but I would perhaps put it a bit more firmly that that is the way we definitely should go, and I think that will be as both Clive and I suggested a doable set of actions.

DR. KASLOW: With no other questions on that, we will move to the last speaker, Dr. Barbara Potts who is the Director of Quality Control Biology at Genentech, South San Francisco. She will be talking to us about Other Porcine Infectious Agents of Concern in Xenotransplantation.

Agenda Item: Other Porcine Infectious Agents of Concern in Xenotransplantation.

DR. POTTS: I have been instructed by two people to keep everybody awake and move this on. So, I will try. To restate this, that Genentech is not involved in xenotransplantation and the data that I will present is actually the result of hard work from my colleagues at the University of Minnesota and also the animals that I studied were from these two sources. Also, X Jay Ming and Sue Emerson have fallen off the edge here at the NIH and my colleague, Dr. Connaughts at Purdue is just a wonderful person, colleagues at ViroMed and also at Mayo Clinic.

So, Mary told me to talk about all the other infectious viruses other than PERV, and the short story is there are a lot and so how could we take a slice through this and how can we focus on what perhaps could be important along with PERV, and so what I have done is I have outlined the viruses and given them either a one, if they are sexually transmitted because artificial insemination was the usual route for impregnating sows in the United States and testing of the semen by PCR for all of the infectious agents that are known to be sexually transmitted should be just a given and then of course, any infectious agent that is zoonotic that can infect humans, we know it can infect humans and xenozoonoses, there are three, are infectious agents that perhaps in a xenotransplant setting where the anatomical and immunological barriers are down could become a zoonosis and then, also, because I am a virologist we all love persistent and latent viruses, but I think those are something to look at, too.

So, I am going to go through this list very fast, and I would just like you to focus on all of the infectious agents that have a two or a three after them, and you can see of course we have PERV that we just talked

about. Swine hepatitis E virus has now actually been moved up to a two. It is known to be a human zoonosis and also the excellent work of X Jay Ming and Sue Emerson we know now that it is not persistent.

So, this work has really moved very fast. Of course we know that swine influenza is a two that can infect humans and encephalomyocarditis I think is a potential xenozoonosis infectious agent. Coronaviruses don't have any numbers after them. They don't look too bad to me. Of course, we have rabies and swine vesicular virus. These are known to be human infections and suipox, well, no, suipox is not known to be infectious and then I realize that I have missed one slide where I have porcine cytomegalovirus and I think that that one definitely we have to look at it as a possible xenozoonotic infectious agent, and it is also persistent and latent.

So, I wanted to just step back a little bit. Being a virologist but now with my new hat on I, also, have microbiology under my watch at Genentech, and I have come to have a full appreciation for the bioburden issues that you face in the industry, and I think some of the things that I would put out to you are that you should have a microbiologist on your Committee; it wouldn't hurt to have a parasitologist, also, and some of my questions for you are the bioburden studies that you are doing now, they are the routine ones that we do for the recombinant protein field. It is a 2-to-5-day culture assay. Are you sure that that is good enough for the bacteria that are known to be in pigs? The USP sterility test is a 2-week test. Have the fungistasis and the bacteriostasis studies been done to prove that you aren't having matrix interference and a real negative? A negative is not a real negative. You really need someone with this microbiology background to be putting these questions out because if you look at the list of bacteria and parasites that do infect humans there are really quite a few. You can see we have toxoplasma, salmonella, Strep suis, Cryptosporidium parvum, Campylobacter, Leptospira, Brucella, Mycobacterium bovis and avium; both are in pigs, Trichinella, Listeria, E. rhusiopathiae and Ascaris and I can tell you that I have been told that the test for this is really, it should be brought into the 21st century. There really needs to be some support for developing some more current tests for some of these organisms

I just want to make sure I got all my points in there on that. Oh, the other point, too, is that with my new hat I realize that mold and yeast may not be detected in a culture where you have bacteria overgrowing. The bacteria will inhibit the mold and yeast detection. So, you really should be looking at two detection methods, one that enhances for mold and yeast and one that enhances for bacteria. So, I would really put some real push back on people who are only doing routine bioburden studies on these tissues, and I had put this in as a reference for you thinking it was going to be larger than it is.

This was published in the April Biopharm, and it is basically a list of all these viruses and the cell lines that they can be isolated on and their detection method, either cytopathic effect or whether or not they hemagglutinate chick, guinea pig or human red blood cells, but I wanted to point out to you that there are three viruses on here that have me worried a little bit, and that is porcine cytomegalovirus really doesn't replicate well in cell culture. Swine hepatitis E doesn't replicate at all in cell culture and the circovirus type 1 and 2, really except for perhaps one cell line that is just privately held doesn't really replicate well in any cell culture. So, you are not going to pick them up in a routine sort of 9 CFR test.

So, I have just put them together here. If I were going to make a slice through the viruses that I think we ought to focus on these are my list, and the swine hepatitis E is an unclassified virus and it causes really no disease in the pigs. So, how are you going to detect a sick pig, and the circo type 1 actually causes no disease, and the circo type 2 has been associated with a post-weaning multisystemic disease, but again it looks like there is some other co-virus that may be indicated in that disease. So, you have a lot of pigs that are circovirus antibody positive for circovirus type 2 and they are very healthy and then you have the

porcine cytomegalovirus in adults causes no disease and there may be some rhinitis in the neonate. So, here you have a group of viruses. They don't grow in cell culture. Because they are not a health issue for the swine industry there are no tests really developed for them and they cause no disease.

So, what do you do? You don't have golden standards and this is really very difficult and these are the viruses that I attacked when I was at the University of Minnesota with all of my help. One of the things I do with viruses is I sort of give them a risk assessment and if you use encephalomyocarditis virus as an example it replicates in human cell lines, in primary cell lines in rodents and in primates.

To me that would be a bad virus and you would want to try to keep it out of your product. So, if you use that as a positive control let us look at porcine cytomegalovirus. I have done some work, and others have reported, also, it doesn't replicate and transfer in human cell lines, but I will present some data today that shows you that it is quite happy in primary human fibroblast cells, and you know none of this has been done, and these are some areas that I would suggest some support for the xenotransplant field is to give some scientists support to address some of these questions. I can tell you these xenotransplant grants have a hard time getting it through the NIH. It is very difficult to get the kind of support you need.

Swine hepatitis D doesn't replicate in cells. However, in some excellent work done by X. Jay Ming and Susan Emerson at the NIH they have been able to show that the swine hepatitis E virus replicates and causes disease, causes hepatitis in rhesus monkeys and by serology there is evidence for swine hepatitis E infection in porcine workers and also X Jay has sequenced a virus from humans that is almost identical to the swine hepatitis E. So, I think in the last year he has moved this from a xenozoonosis to a known zoonosis, but there is good news at the end of the story on that one and then circovirus is really difficult to study because it really doesn't grow and hasn't really been studied very well in primates.

There is some early serologic evidence suggesting that there may be a circovirus in rodents. So, to start out I needed to develop some ELISA assays to be able to just monitor these animals. So, being a practical person, the first thing I looked at was the kits that are available to detect at least hepatitis E antibody and porcine cytomegalovirus antibody in humans and just adapt them to the pig, and I went through quite a few kits, and these are the ones that work. For swine hepatitis E you can use the antigen HEV antibody kit. All you do is change the detector antibody and the antigen is a peptide made to the human HEV. It is a Chinese HEV to the ORF 2 and the ORF 3, the open reading frame 2 and 3. Circovirus type 1 and 2 there was nothing out there. So, we developed a peptide based ELISA that detected, that was to the ORF 1 which has an 82 percent homology between the two viruses. So, it detects antibody to both and then developed another one to the ORF 5 which has no homology to type 1. So, it only detects type 2 antibody.

This assay will be made into a kit pretty soon for people to have access to. Porcine cytomegalovirus went through all the commercial kits and the only one that worked was the Trinity Biotech CMV EIA kit. Changed the detector and I get nice detection of antibody, and the antigen here is to the early, late cytoplasmic antigen structural and non-structural proteins. It is basically a semipurified cell lysate.

This is supposed to be Taq here, I am sorry, Taqman PCR. We developed the targets to swine hepatitis E to the ORF 2 because it has the most conserved region and X. Jay Ming had already shown that this region, PCR to this region would pick up all of the hepatitis E isolates.

Circovirus type 1, designed an amplicon to the ORF 1 because of the 82 percent homology and porcine cytomegalovirus designed primers to the DNA polymerase because it was the only sequence that was in Genbank and luckily it worked pretty well.

Positive controls, I used fecal sample from swine hepatitis E infected rhesus monkey. This was provide to me by Susan Emerson and X. Jay. For circovirus type 1 we used PK15 cells. For type 2 we used tissue and then Dr. Conn provided me with the porcine CMV infected cells. So, I could use that as the positive control for that assay and then the areas of the animals where we could isolate the virus and be able to detect it by PCR in a reproducible manner, fecal samples for swine hepatitis E not peripheral blood. Circovirus, mesenteric lymph node, I thought if you really want to detect it that is where you have to go, not peripheral blood.

CMV circulating mononuclear cells, almost every adult pig that I looked at had circulating mononuclear cells that I could detect by PCR. I don't think I need to tell you about what CMV is, but porcine CMV is very similar to human CMV. There is prolonged viral shedding. There is a high rate of infection found. Dr. Conn did a lot of this work in the 1970s and has been extremely giving to give me all of the cell lines and the virus-infected cells and he gave me permission to make stocks of these and submit them to ATCC. So, Bob Haye at ATCC can get you this virus and the cells for growing this virus.

There is transmission transplacentally, during delivery and through maternal milk and as Marian pointed out human CMV is an important hazard of organ transplant, and I would worry about porcine CMV.

Just a little bit about the virus that Dr. Conn provided to me. It is a bear to work with. Ten infectious units per ml, just minimal cytopathic effect and we basically detected it primarily by PCR, but we, also, detected it by immunofluorescence using a sera that Dr. Conn provided to me.

There is supposed to be a line here just basically showing the titration of the positive antisera that Dr. Conn provided to me on the Trinity ELISA kit, and these are the negative controls down here. So, using this Trinity assay that I have adapted to porcine serum I collaborated with Rexanne Stuve who has gnotobiotic who provides gnotobiotic animals to the research field and it was good that Dr. Swindle went ahead so he could tell you how these animals are taken.

They are taken in a bubble that has been fumigated. Everything that goes into the bubble has been sterilized and the plumbing on the sheep; I used to do sheep, the plumbing on pigs is really quite nice because each piglet has its own placenta and its own connection. so, you have up to 12 separate little experiments in one sow that you can study. Also, in pigs there is no transmission of maternal antibody to the piglets. They get all of their maternal antibody from colostrum.

So, if there is any antibody in these piglets when you take them by cesarean section that means that they have had an infection in utero. They are immune competent around 70 days, I think, right, gestation, and so they are really a nice way to study. What I use these for is to study the natural seroconversion of these piglets in three sows and I run this assay at a 1 to 40 dilution and then let the ODs give me an indication of the titer. The sow here has an OD of around 3. That would have titered out to about 1 to 10,000 if I titered it out, and the IFA was about 1 to 4000.

So, you can see that the newborn animals were all negative, all below the cutoff and then by 1 week we have two animals that have already seroconverted, and then by 2 and 3 weeks all of these animals are starting to get rising titers to CMV, and I might back up and say that even though the gnotobiotic animals are taken in a really very precise manner, they still do get some maternal blood and they can aspirate some maternal blood, and my guess is that may be how they are getting the infection since these sows are circulating virus in their peripheral mononuclear cells and the other sow we had one animal here that may have been congenitally infected. It was right at the cutoff. We repeated this many times and there may be one that was congenital, and you can see again by 3 weeks of age all but one of these animals had

seroconverted to CMV and again this sow here had this huge titer, and by 1 week of age all of these animals had seroconverted to CMV.

So, I think that this is a challenge for the field to get CMV-free animals. We developed a Taqman PCR directed to the DNA polymerase region of porcine CMV but I wanted to just ask could any of the human CMV tests, PCR tests or the bDNA or the RNA DNA hybridization tests detect porcine CMV. Perhaps there is a way to use the established tests and play them off of each other so that you could look at patients. So, of course, the cell culture in the field isolates were positive in our Taqman PCR. ViroMed ran the cell culture which had the highest virus titer and it was negative using the PCR assay which is targeted to the immediate early region.

I sent it to a colleague at Chiron. She ran it in the bDNA assay. bDNA assay detected both the cell culture and the field isolate of porcine CMV, sent it to a colleague at Digene who did the CMV DNA RNA hybridization study and they did not detect the virus by this method. So, there may be a way for the field to play these two off if you wanted to ask do you have porcine CMV in the face of also human CMV in a patient.

So, just to sort of summarize, Mary wanted me to tell you the pros and the cons of assays is that ELISA just never lies to me. If you make a tight enough assay that is sensitive it is always going to tell you when a virus is on board. PCR the sensitivity is always in question and in my hands the Taqman PCR wasn't as sensitive as nested set PCR with my colleagues at Mayo Clinic and also colleagues at the NIH when we did comparisons.

So, we looked at 23 newborns. They were all negative both in ELISA and PCR, but we took this, looked at this one pig from 6 to 11 weeks, and they were positive in ELISA but I just didn't pick them up in my PCR assay. So, it is really not sensitive enough. I wouldn't use this as a final product test. I would probably go to nested set PCR with known copy number for final product testing and then we looked at an animal, another animal from 6 through 12 weeks. We were positive in both but you know I had one adult here who came up negative and then I had multiple adults who both came up positive and negative, and I just had one overhead to show you of the replication of porcine CMV in primary human fibroblast cells.

The dogma was that CMVs were species specific, but when I looked for evidence of the dogma I couldn't find any publications. So, what I did is I just treated the porcine cytomegalo-infected cells the same way a clinician would do doing a primary isolate of human CMV from say a child. You do a bead disruption of the cells, spin out the cell lysate and then co-cultivate onto indicator cells, and I used primary human fibroblast cells, and what I found is that it was very difficult for me to replicate porcine cytomegalovirus in porcine primary turbinate cells. I got 10 infectious units per ml, but the same inoculum put on primary human fibroblasts this virus took off. Within 10 days it grew much better in the primary human cells than it did in the turbinate cells and just to convince myself that somehow I wasn't putting turbinate porcine cells in there and getting kind of fooled I used a monoclonal antibody to a subunit of one of the cell adhesion molecules that was specific for human cells and did dual immune labeling. So, I have a monoclonal here to CD49B showing me that I have mostly human cells and then dual labeled with the porcine anti-CMV antibody. So, you can see that these are human cells that are infected with porcine CMV and it did not, this antibody did not react to the porcine cells.

I am going to move very fast here. Just quickly about the circovirus, it is really small. It is really stable. I think it was 2 months ago in Biopharm there was a study where it is resistant to gamma radiation and so it is a bit of a worry. It has a high infection with type 1 and there is this disease associated with the

presence of type 2, but it has this limited host range. There is a circovirus type 2 reported in cattle sera.

So, I designed two peptides to try to detect the type 1 and the type 2. Basically this one would detect both and this type 2 because its design to the orph 5 would only detect the type 2, and if I compare just some animals that I just got from this one farm where there were no special conditions all of the newborn animals were negative for both. What you can see is that the universal, the type 1 and type 2 even by 3 weeks we have got quite high ODs and then also the type 2 is coming up.

So, what I wanted to do was look at this early period in here if there was a way that we could break this, and again it was the same animals that I studied for porcine CMV. I also studied for circovirus and you can see that the conversion took about a week longer than for CMV but by 2 weeks of age we have seroconversion here of all of the piglets from this sow half of the piglets from this sow and most of the piglets from this sow, and I might back up that the method for detecting circovirus that most labs are using is an IFA test and the sows here that have these very high ODs would have around a titer of about again 1 to 10,000, would only score weakly positive in the IFA assay. So, the peptide assay is much more sensitive.

Just quickly on the swine hepatitis E virus, it is a little bit larger, but it is still resistant to physical-chemical treatment. It is closely related to human hepatitis E and it now is being considered by the CDC as an emerging zoonotic disease, and this is primarily because of the excellent work done by Sue Emerson and X Jay Ming at the NIH. So, they were kind enough to share with me their sera from an experiment where they took swine hepatitis E virus and inoculated three pigs and followed them for 6 weeks and so this is comparing one of their recombinant protein ELISAs and comparing this ORF 2 and 3 ELISA that is from Antigen Biotech and you can see if you just look down here that there is complete correlation. This animal didn't seroconvert until later but in this one here we had one animal that converted 1 week earlier than in this assay.

So, we felt pretty comfortable. At least we had a golden standard for hepatitis E and so we felt pretty comfortable with going with this peptide ELISA. So, we worked with David Theis who has a conventional farm but provides animals for xenotransplantation and he designed this experiment where he did early weaning and off-site rearing. Hepatitis E is an oral fecal route of transmission and what we found is if we looked at animals all the way out to 17 weeks of age they were non-reactive in two of the NIH assays and non-reactive in our assay. So, this virus can very easily be removed from your donor pigs by doing early weaning and off-site rearing. This is conventional farm, no gnotobiotics or anything and again we looked at the animals in the CDC facility which are the gnotobiotic animals that are taken by C section and colostrum deprived and again they were all non-reactive.

So, in summary I think that swine hepatitis E is easy to get out and I don't think you need to worry about it if you have the right kind of animal husbandry. I think this virus and the serology certainly is a good indication of no virus being present in the animals. I might back up. I tried really hard. One of my colleagues tried really hard with many, many fecal samples and this virus is not persistent. It is shed transiently and we were never ever able to catch it in the fecal material of just positive animals. You really need to catch it just before they seroconvert. So, this virus is in and it is out, and if you have the right animal husbandry you can keep it out.

Circovirus is going to be difficult. The fact that there was like a week delay in the seroconversion, this particular facility does parasitic fumigation. Then they take the individual animals and for 1 week they put them in an isolator that has been decontaminated but then they move them with two other animals and those cages are only washed. They are not autoclaved and circovirus is really resistant to most

inactivation procedures and then porcine cytomegalovirus you know I am sure that we will be able to get it out of the herds, but I think that we really need some serious study from the CMV scientists.

Thank you.

(Applause.)

DR. KASLOW: Are there questions for Dr. Potts?

DR. ALLAN: Barbara, that was very good. I think it is important to talk about viruses other than PERVs because we are looking at a spectrum of potential infections. The question I had was more technical than anything. The litters that you worked with when they were, those were all C sections, and how were they exposed? They were put back with the sow?

DR. POTTS: No, that is just environmental.

DR. ALLAN: So, they just receive formula then?

DR. POTTS: Yes, no maternal antibody. I think with the CMV because it is hard to get maternal blood, you know, some maternal blood gets on their faces and if they aspirate a little of that, that is how we experimentally infect them as we just aspirate it into their nostrils, and the fact that it was so early I suspect that is what is going on with CMV. With circovirus I think it is environmental, and I think with the right kind of decontamination autoclaving I think that you may be able to block that transmission. That was just a natural transmission of the virus.

DR. ALLAN: Did you look at the circovirus to see if, I cannot remember whether it infected human cells?

DR. POTTS: I haven't done that. This is a virus that grows in one particular cell line and the only one I believe is the one that X. Jay Ming has developed. He is doing that right now and I might give a pitch for him. I think that we need to put this virus into some chimps and see if this is something we need to worry about, and he got serious push back on that when he requested it. So, I think we need to give some support to our scientists to be able to do some of these studies to just see if this is an important virus or not, but X. Jay is at West Virginia University now and is carrying these studies out.

DR. MICHAELS: Barb, nice studies. I just wanted to ask do you think or maybe Mike has some ideas on this, too, would a hysterotomy-derived pig then be better than a hysterectomy-derived I mean in terms of not having the blood transmission into the nares and not getting the viral infection and then the second question was is there any chance that some of them were in-utero infections? Did you try to do any isolation of the virus from those newborns?

DR. POTTS: The way that these animals were taken is the individual placentas were taken out and put in so that each individual placenta was processed but it is still very difficult to not get some maternal blood. Growing CMV on primary turbinate porcine cells is very difficult when I have a known virus stock. Now that I know that this virus grows on primary human foreskin I think someone ought to do those studies. The fact that there was one animal that was on the borderline of seroconverting, if they had been infected in utero I think I would have expected some more antibody present in the newborns, but I think that you need to look directly. We did do PCR on 24 newborns and they were all negative but our PCR assay wasn't exquisitely sensitive.

DR. SWINDLE: Just a comment on your question on the various surgical techniques. Speed is going to become a real issue on that having done it because you can have a huge uterus with a dozen animals in there and they will be really depressed by the time you get to the last one. So, I don't think it is too practical in my experience.

DR. POTTS: I did try some ganciclovir and it didn't block it but I probably didn't do it as well as someone who is in the CMV field. I used the same concentrations that would be used in a clinical study. One thing I forgot to mention is on the microbiology side there are some rapid ID tests out there available now. There is one called Cheminex RD and another one Microstar that they might be looked at so that there could be more rapid detection of bacterial contamination of tissues if you cannot freeze them down before you transplant them.

DR. KASLOW: Just to follow up more generally are there other things that you heard from her talk that would suggest any barriers or problems that are not being faced in your whole system of breeding and development of the litters and the whole stock? Anything you heard from all of that that would suggest that we are missing something?

DR. SWINDLE: Not really other than the few things I pointed out like you are going to run across congenital defects and other non-infectious congenital diseases which I never hear addressed in the last 5, 6 years.

DR. KASLOW: I think I took the same message, too. Both neoplasia and congenital disease are clearly phenomena that can be infectious in nature. We have good examples of that and I think the whole monitoring system that we would consider developing needs to take that into account, too, in some way or another.

Other questions? Yes?

DR. SYKES: I was just wondering if you had tried to infect any non-human primate fibroblasts with porcine CMV like baboons for example?

DR. POTTS: I just did Vero. That was the only one that I did. So, I didn't take it further.

DR. SYKES: The reason I ask is in our research center David Cooper with Jay Fishman has been looking at this issue in heavily immunosuppressed baboons that received porcine organ xenografts. They have been looking for porcine CMV infection and at baboon CMV and as far as I know there has been no evidence of transmission of porcine CMV, but it would be of interest to know what has shown up in your assay of that species.

DR. POTTS: And you know this may be the same story as with PERV but it needs to be played out and it needs to be played out by some CMV experts, and that was one of the reasons I submitted the virus and the cells to ATCC. So, someone else could get going on that.

DR. KASLOW: Other questions or comments on this presentation or if not on any of the presentations? We can open this up for a few minutes of further comment if there are any comments from anyone else.

DR. BERGER: This is really for Dan. It is really a two-part question. How much weight do you put in the limited studies that we have had on PERV based on the fact that they were after the fact? They weren't controlled studies and then secondly, has the research that has been done been so concentrated on

PERV that we have left out looking at other potential problems? After all PERV was an unknown virus just 3 or 4 years ago.

DR. SALOMON: One fact change, it was discovered 20 years ago and didn't cause any disease in pigs, and so everyone lost interest in it, but it was really 5 years ago that everyone got excited again about it. So, to that extent you are correct. Yes, I think we probably, I mean did we over concentrate on PERV? No, in the sense that it was such a clear and perfect model to focus on initially because it was an endogenous retrovirus. It was definitely going to be there, if it was going to produce disease and the kinds of diseases that it would produce could potentially be both important to the patient and have a public health impact. So, I think that was quite appropriate and we learned a lot about the design of the studies and what sorts of things we needed to do and how to do it, but yes, you are, also, right that none of us working in the field are suggesting that PERV was the last thing to worry about. Robin Weiss had some really nice data he presented at Scripps. I am 99 percent sure it is not published yet. At least I have missed it and he was using degenerative PCR primers for reverse transcriptase and came up with two or three other non-PERV retroviruses endogenous in the pig. So, I think your points are really well taken.

DR. PATIENCE: Could I jump in there, Dan? We published those results 2 months ago in *J. Virol.*, but you are more up to date in other journals. While the microphone is on I guess I would direct a question to Barbara if I could and if she is with us.

Barbara, it is Clive. I just wondered if you were working in a company that was working towards xeno and you have got that immense knowledge at your grasp now with respect to non-endogenous microorganisms, do you think that all of the microorganisms that we know about it is achievable to remove them from the herd?

DR. POTTS: As scientists we are so conservative, you know, the public should feel very comfortable with us. I really do. I think that it is just paying a little more attention to it and you know getting really sensitive assays so that you know right away that at 1 week you have lost out or not. I think that it is achievable. The swine industry is very sophisticated and I think they are really eager to work with the field. So, I think it is doable. If you guys can get PERV to not be a problem, I think, but you know like the CMV needs to be studied by some CMV people who have seen it devastate children so they have that full appreciation of its problems, and it may not be a problem, you know. It may be like PERV. It may not come up.

DR. SCHECKLER: As we anguish over PERV and all of the porcine bugs that we have heard about today I want to mention the fact that there are two recent examples that I am familiar with where epidemiology, science, biology are extremely well known and the public and even professional reaction to them has not been influenced by that science.

The first example is from Ohio where two high school kids died of meningococcal disease and a third one 15 miles away had a similar type C meningococcal disease and 40,000 people in that community received one dose of Ciprofloxacin as a preventive prophylactic effort. I don't know where the public health system went wrong or what happened. I have talked to several people about it but that was totally irrational. People were afraid to share pens in the line waiting for their dose of antibiotic. There is absolutely no reason why all of those people got meningococcal prophylaxis. This was just a month ago, but that happened and that happened right here in the United States and that happened in a midwestern state where we are all kindly and friendly, but totally ignored all of the science and dealing with the fear in the community because of the deaths was the problem and good science made no difference at all.

The second example that I have is we think about where we don't know everything in the issues of xenotransplantation is a zoonosis. It is a case of rabies. It is a case of rabies in my hospital in Madison, Wisconsin where we hadn't had a human case of rabies since 1959, and the CDC had done a nice publication in the *Annals of Internal Medicine* looking at about 32 cases over the last 15 years or so published it in 1999 and the average number of people that got post-exposure prophylaxis was 54 per each case. In our setting we had a man that came in with no history of an animal bite fledged in from a rural hospital with an encephalopathy who continued to get worse, require pulmonary support and eventually that was withdrawn and he died and nobody really knew what he had. Someone had done a test for rabies antibody which was negative. It turns out to be the wrong test to do and 3 weeks later as hospital epidemiologist I got a call from a nurse, "Dr. Scheckler, do you remember that guy up in the medical intensive care unit for 10 days?"

"Yes, the residents even discussed him with me." "Well, he had Negri bodies in his brain, and they are sending the tissue down to the CDC. We think he may have had rabies."

What is the problem? The problem was that we had a whole bunch of doctors, nurses, respiratory therapists exposed to this fellow over a 10-day period and they didn't know that he had rabies. Now, if the world had been perfect and everybody had been using the proper precautions, what they are supposed to, what is on the books then no one would have had any kind of exposure. The reality is the risk to a health care worker of a secondary spread of rabies is virtually non-existent. There has never been a case. I have talked to physicians in India that used to take care of wards full of human rabies cases without any particular precautions and there had never been a case, and that is the area of the world where there are thousands of cases a year, and yet we wound up having 16 of our nurses and respiratory therapists and one physician get post-exposure prophylaxis where probably only three met the best guidelines from the CDC and from our veterinary colleague in the public health that needed it, and these are very good professionals and actually I wound up feeling fairly good about that because it wasn't 54; it was only 16. So, I thought we had beaten the odds. but here is a whole bunch of professionals, very sophisticated people who simply could not remember what their level of exposure was and whether they had had a bleeding wound on their hand when they touched this person's saliva or not, and we know the risks. We have excellent epidemiologic data and human-human transmission of rabies is virtually non-existent and fear will drive out science every time, and to be honest with you I think that is what is happening in the UK and Europe right now, that there is a fear of the unknown and because of the mad cow disease that alone that has killed some younger people has made people extraordinarily worried.

So, the lesson here I guess relates to some of the things that we are going to talk about tomorrow but it is a real disconnect as we think that we can develop very good science and come up with recommendations which should be based on good science. There are human and political and fear dimensions to this which we probably can never overcome, and that is my sermonette for today.

DR. KASLOW: Any other questions or comments?

Dr. Potts?

DR. POTTS: Just to keep everybody here, perhaps then we aren't doing our job in getting our science out to the general public and you know there are many reporters, newspaper reporters who are excellent and give really well-balanced reports. We had one in Minneapolis. She did an excellent job. She looked at both sides and you know she really tried to educate the people in Minnesota, and I know that got picked up on the wire and ended up in a small article in the *New York Times* and on the West Coast, but I think as scientists we are shy to talk to the press. It looks like we are trying to get attention or something

but I think it is something maybe we need to do is get this information in a way that the general public can understand it and get it out there to the newspapers, a nice article in Newsweek, I mean you know, there has to be some push to keep everybody educated.

DR. KASLOW: In Minnesota, of course, all the children are slightly above average. So, I think there is an opportunity there that we may not all have, but thank you.

DR. POTTS: That is what they think.

DR. KASLOW; Any other last minute or last ditch comment from anyone? Dr. Vanderpool, then will close the meeting for us.

DR. VANDERPOOL: Thanks, Dick. I praise Dick Kaslow for facilitating this excellent meeting. We came here after deciding that we should explore the infectious disease issues related to xenotransplantation and I want to praise Mary Groesch for putting together an excellent program, praise each of you speakers and thank the Committee members for sharing, several Committee members for sharing their expertise concerning this, and I can speak I think for the non-scientists to say that I am greatly heartened to have the scientific expertise we have on this Committee.

I wanted to just say something in terms of the summary of the day and present a challenge. The summary is that we have had an excellent and well-organized program and I am going to briefly look at this in the chronological order in which it was initially intended which was rudely interrupted by of all things the weather. Weather ought to conform to SAC meetings much better, but we first talked about introduction of cross-species transmission of infectious disease and had excellent presentations on these. Jon Allan talked about the dynamics of xenotic transmission, Brian Mahy giving us a world tour of the inventiveness and seductiveness and destructiveness of viral infections across the world and over time.

We then heard Marian Michaels demonstrate that allotransplants have been also beset by infectious disease issues and I think one of the heartening sides of her presentation is that you cannot cover every single base. You discover new things over time and you respond to them as you go along. We will have to make a judgment call concerning how much we have to cover and how much we will leave to be responded to over time.

We then had an entire session on porcine infectious agents, again, three excellent presentations by Clive Patience and David Onions and Dan Salomon presenting fascinating and important very up-to-date information about PERV and what is being done about it and the hope or promise that we will be able to in the foreseeable future have pigs that are PERV infection free with respect to human beings.

Then I have to revisit our analogy again. Dan presented a fascinating view of retrospective studies and asked us whether PERV is a sleeping snake or a dead snake and we indulged in very unprofessional language about how the snake might be badly wounded and in a semi-comatose state with Dan saying at the end he didn't like snakes period. So, he would rather go without PERV altogether which I think we can surely agree with.

We then had, we just had but I am talking about the order of things, Barbara presenting intriguing and for me newly informative data about other types of infections and I think that there certainly is a further discussion with Michael Swindle about the degree to which all of these issues are taken in consideration in the colonies that are being developed.

Now, after we did all that in terms of the actual logic of the program which Mary Groesch fully foresaw we had a presentation by Eda Bloom about the FDA requirements and recommendations, and our question concerning that is are these adequate for the issues we have discussed and I think each of us should feel a responsibility to review her outline and to read those guidelines and see whether we have further recommendations with respect to those guidelines and then Michael showed us a walking tour of an infection-free colony. I appreciate that, Michael. I wanted to be able to walk one of these personally, but you got me as close as I have ever been to actually walking through one of these facilities, and I am glad to see that they could root basketballs around their cages and sniff at each other, and then of course or Committee member all along but thankfully present with us today, Tony Lubiniecki talked about what industry has been doing and again I think a lot of this was heartening in the sense of how the tests and the analyses are ever more sophisticated with the sobering message that you can't do all 100 and whatever tests, but you can choose 20 to get the information that you want.

So, we have covered a range of very important issues. I think I can think of no other really significant bodies of data in addition to the ones we have had and this is the challenge I will present to us all.

Tomorrow from 9:15-10:15 I am to moderate our discussion of infectious disease risk in xenotransplantation ethical, social, legal considerations. I want to revise that title to say, "Scientific, clinical, ethical, social and legal considerations," the scientific ones being do we have recommendations in the light of what we have heard regarding what more if there is more that should be done regarding the control and study of infectious disease issues and thus take that seriously.

We have a number of persons on this Committee with public health expertise, and I would like since it is late in the day not to open that subject up for today but tomorrow to have those of you with public health expertise, Louise Chapman and certainly a number of the members of the Committee to talk about whether you have continuing worries such that you would want to see certain things done or certain recommendations made, but we, also, have those ethical, social and legal considerations and one could say that we are supposed to cover all the bases, but we don't have to cover every one of them to be secure and then but Robin may come back and say, "What happens when one person gets sick, and the litigation starts and you didn't cover everything just right?" and I don't think it would probably do in court to say, "But you should have heard Brian Mahy's message about how viruses are just all over the place and this just happens to be a rogue virus in a xenotransplant setting."

I am not sure we can do that. So, we do have a number of issues to work off of in terms of these infectious disease issues, but we have covered a great deal today and I thank everyone for her and his participation, the public for being here and look forward to seeing you all tomorrow.

Thank you.

(Applause.)

(Thereupon, at 5:28 p.m., a recess was taken until 8 a.m., the following day, July 3, 2001.)