DEPARTMENT OF HEALTH AND HUMAN SERVICES

FOOD AND DRUG ADMINISTRATION

CENTER FOR BIOLOGICS EVALUATION AND RESEARCH

BIOLOGICAL RESPONSE MODIFIERS ADVISORY COMMITTEE

XENOTRANSPLANTATION SUBCOMMITTEE

OPEN

Thursday, June 3, 1999

8:30 a.m.

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Ms. Abbey S. Meyers

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Mr. Antonio Benedi
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GUESTS

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Ralf R. Toenjes, Ph.D.
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C O N T E N T S

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P R O C E E D I N G S

Welcome

MS. DAPOLITO: I would to welcome the subcommittee participants and guests, and all here, to this meeting of the FDA Xenotransplantation Subcommittee. I am Gail Dapolito, and the Executive Secretary of the subcommittee, and a designated federal official for today's proceedings.

I would like to begin by stating today's meeting will be conducted as a subcommittee meeting of the Biological Response Modifiers Advisory Committee. As is evident, this is a subcommittee on xenotransplantation. This was listed in the Federal Register Notice.

There are two notable differences between a subcommittee meeting and a full advisory committee meeting. One is that there are only two standing advisory committee members present at the table -- one right now. They will be introduced to you in a moment. The other difference is the that report that will be furnished by the subcommittee following today's discussions will be approved by the parent committee, the Biological Response Modifiers Advisory Committee. Other than that, today's discussion will proceed as would a full advisory committee meeting.

At this time, I would like to introduce today's participants seated at the table. I will begin on my left: Dr. Prem Paul, Iowa State University; Dr. John Coffin, who

will be joining us soon, Tufts University School of Medicine; Dr. John Conte, the Johns Hopkins Hospital; Mr. Antonio Benedi, Transplant Recipient International; Mr. William Lawrence, United Network for Organ Sharing. Mr. Benedi and Mr. Lawrence are participating today as patient representatives of the subcommittee. Dr. Robert Michler, Ohio State University Medical Center. Next we have Dr. Claudia Mickelson, Massachusetts Institute of Technology. Next to Dr. Mickelson is Dr. Ralf Toenjes, Paul Erlich Institute; Dr. Jonathan Allan, Southwest Foundation for Biomedical Research; Dr. Marian Michaels, University of Pittsburgh School of Medicine, Children's Hospital of Pittsburgh. Going around the table, Dr. Martin Hirsch, Harvard Medical School, Massachusetts Medical Hospital; Dr. Richard Kaslow, University of Alabama at Birmingham; Dr. David Onions, University of Glasgow; Dr. Harold Vanderpool, University of Texas Medical Branch; Dr. Daniel Salomon, the Scripps Research Institute. Dr. Salomon is a current member of the Biological Response Modifiers Advisory Committee.

Next to Dr. Salomon when he arrives; he is running a little late; Dr. Hugh Auchincloss, Harvard Medical School, Massachusetts General Hospital. Dr. Auchincloss is the chair of this subcommittee and a current member of the Biological Response Modifiers Advisory Committee and in his absence, until he arrives, Dr. Salomon will be participating as the

chair of the subcommittee. Dr. Leroy Walters, Georgetown University, Kennedy Institute of Ethics; Dr. Nicholas Lerche, University of California; Ms. Abbey Meyers, National Organization for Rare Disorders. Ms. Meyers is a recent past member of the Biological Response Modifiers Advisory Committee. She is participating today as the consumer representative of the subcommittee.

Proceeding around the table, Dr. David Sachs, who also will be joining us later, Harvard Medical School, Massachusetts General Hospital; and Dr. Steve Woodle, and he will be coming in later this morning too, University of Chicago. Dr. Woodle also is a past member of the parent committee. Dr. Mary Groesch is representing the National Institutes of Health. Dr. Louisa Chapman is representing the Centers for Disease Control and Prevention.

The FDA is represented today by Dr. Louis Marzella; Dr. Karen Weiss is not here; Dr. Carolyn Wilson; Dr. Eda Bloom; Dr. Philip Noguchi; and Dr. Jay Siegel.

There are two participants listed on your roster who are unable to make it this morning, Dr. Ronald Desrosier and Dr. Manikkam Suthanthiran.

As a courtesy to the participants and to your neighbors in the audience, we ask that cellular phones be turned off in the conference room. Please go outside in the lobby if you would like to use your cellular phone. And, we

ask that pagers be set on the silent mode.

A couple of final announcements -- the questions for the committee discussion today may be found in the stapled materials where the agenda is on the front page. In addition, or the speakers here today there is a speaker ready-room across the hall if anyone needs to use that.

As you have noticed, on this floor of the hotel we are having a little bit of an air-conditioning problem. They are trying their best to keep this room cool. So, if you will bear with us, we hope we can keep it as comfortable as possible.

I would like to make just one final request, the FDA isn't known for having a whole lot of money so, unfortunately, we can only provide coffee and refreshments for members. I would ask you to reserve the side table for members.

Conflict of Interest

Thank you. Dr. Salomon, with your permission, I will read the meeting statement. The following announcement is made part of the public record to preclude even the appearance of a conflict of interest at this meeting. Pursuant to the authority granted under the Committee Charter, the Director, Center for Biologics Evaluation and Research, has appointed the following participants as temporary voting members: Dr. Hugh Auchincloss, Jonathan

Allan, John Coffin, Martin Hirsch, Richard Kaslow, Nicholas Lerche, Abbey Meyers, Claudia Mickelson, Prem Paul, David Sachs, Daniel Salomon, Harold Vanderpool, Leroy Walters and Steve Woodle. In addition, Mr. Antonio Benedi and Mr. William Lawrence have been appointed as temporary voting members.

Based on the agenda made available, it has been determined that the agenda addresses general matters. All financial interests in firms regulated by the Center for Biologics Evaluation and Research which have been reported by the subcommittee participants and consultants as of this date have been considered. In accordance with 18 USC 208, the following participants have been granted a general matters waiver which permits them to participate in the committee discussions: Dr. Martin Hirsch, Dr. David Onions, Prem Paul, David Sachs and Daniel Salomon. Also, the waiver approved for Dr. Hugh Auchincloss on July 10, 1995 is applicable for this meeting.

The following guest participants have been invited to participate in today's discussion by the Chair of the Xenotransplantation Subcommittee: Dr. Robert Michler, Dr. Marian Michaels, Dr. John Conte and Dr. Ralf Toenjes.

With regards to FDA's invited guests, the agency has determined that the services of these guests are essential. The following interests are being made public to

allow meeting participants to objectively evaluate any presentation and/or comments made by the participants. Dr. Louisa Chapman is employed by CDC. She and other CDC employees are involved in collaborative research monitoring for evidence of porcine endogenous retrovirus infection, and performing confirmatory testing in exposed persons or firms that could be affected. No financial support is received.

Dr. John Conte is Director of Johns Hopkins Heart and Lung Transplant Program. Colleagues in this program are involved in trials supported by a firm that could be affected by the discussions. Some of Dr. Conte's patients could be enrolled in these trials.

Dr. David Cooper reported that he receives consulting fees from Biotransplant, and is a science advisor for Biotransplant.

In the event that the discussions involve other products of firms not already on the agenda for which FDA's participants have a financial interest, the participants are aware of the need to exclude themselves from such involvement, and their exclusion will be noted for the public record. A copy of the waivers is available by written request under the Freedom of Information Act.

With respect to all other meeting participants, we ask in the interest of fairness that they address any current or previous financial involvement with any firm

whose products they wish to comment upon.

I will turn it over to you, Dr. Salomon.

DR. SALOMON: Well, I would like to welcome everybody today, and the meeting begins with Dr. Jay Siegel making some introductory remarks for the FDA.

Introduction

DR. SIEGEL: Thank you, and I would like to welcome the members of the committee as well as the members of the audience. The FDA and its sister agencies in the Public Health Service, NIH and CDC, have been committed through the process of dealing with xenotransplantation to seeking the best in scientific input, as well as to having an open process with public discussion of critical issues, and we look forward to this meeting as helping to fulfill both of those goals.

This is a particularly important and also challenging area of biological product development. At this particular meeting we have what I consider to be a rather varied and interesting agenda that, as often is the case with xenotransplantation, spans the broad range of scientific and medical disciplines that come into play in conducting and carrying out this research.

We will have three topics, the first being an update on issues of porcine endogenous retrovirus, a topic we have been discussing with this committee and in other

public fora for a little over two years now, is that right? And, an update on additional findings and recent policy developments and seeking further input in that area.

We will be discussing PHS and FDA policy guidelines regarding xenotransplantation, something we have also had in development and discussion for I guess going on for five years now. Specifically, we are going to be touching on an important aspect of that, which has perhaps not been well discussed till now, which is the impact of some of the varied approaches to xenotransplantation and varied characteristics of xenotransplantation protocols on how they may impact appropriate public health and policy measures to ensure that they are conducted in a safe manner.

Tomorrow we will have what promises to be a very informative and intriguing discussion on issues in clinical development of solid organ xenotransplantation.

So, again, I am really excited by this agenda; I am looking forward to the advice we will receive, and I welcome you and all and thank you all in advance for your participation.

DR. SALOMON: Thank you, Dr. Siegel. Well, that officially then introduces us to the first topic, which is an update on porcine endogenous retrovirus. I have been reminded that before opening this part, because this is a public hearing and in the nature of a free and open exchange

with the public, it is appropriate at this point that if anyone in the audience would like to make any comments, any introductory comments or questions to the group that they may step forward at this point. There were no formal previous requests but, again, that doesn't prevent anyone from stepping up at this point.

I guess this is the point in the wedding where you go, "if there are no objections to this union," --

[Laughter]

-- of course, the mike is not going away; the public is more than invited or encouraged to step up at any key point and contribute to this discussion. That would be great.

Then, we will open the first topic, porcine endogenous retrovirus update, and the introduction and a perspective will be given by Dr. Carolyn Wilson of the Office of Therapeutics Research and Review for CBER.

Topic I: Porcine Endogenous Retrovirus Update

[Slide]

DR. WILSON: Again, I am going to also welcome the members of the committee and the audience. As already introduced, this first session of this two-day meeting is meant to update the committee on pertinent scientific and medical data on the in vitro and in vivo biology of porcine endogenous retrovirus.

[Slide]

Although there have been a number of scientific publications of data in this area, we also, at the FDA, have received data from sponsors and that data has come in, in response to a letter that was issued in October of 1997. At that time, all sponsors of porcine xenografts were placed on clinical hold pending, first, the development of assays for detection of infectious retrovirus in porcine xenografts. They were asked to submit that data for review by the FDA; secondly, to develop assays to detect evidence of infection by this retrovirus in recipients of porcine xenografts. Again, this data was asked to be submitted for review. In addition, they were asked to develop contingency plans in the event of a positive result in the testing of patients and to update the informed consent documents in order to acknowledge potential risks presented by the porcine endogenous retrovirus.

It is the data that the FDA has received in response to the first two points of this letter that will be the focus of today's presentations and discussion. In particular, when this committee last met in December of 1997, t here was a lot of interest expressed by committee members to hear what the results of monitoring of patients were going to be. So, you will see that on the agenda there are a number of presentations by sponsors of their results

from looking at patients, and there will be an emphasis in the discussion section on this topic as well.

[Slide]

Since Dr. Coffin isn't here yet, I will proceed with my talk and then he will talk after me. So, the purpose of my talk is to provide an overview on the data that FDA has received from the sponsors in response to that letter. In addition, I will try to also incorporate data that has been published in the scientific literature and, in the last few minutes of my talk, discuss some data that we have generated here at CBER.

Of the initial ten INDs that were placed on clinical hold in October of '97, six have been able to adequately address the issue posed in that letter. The data that they have presented to the FDA on their infectivity assays and their assays to monitor patients is what the focus of my talk will be today.

[Slide]

This is a summary of the data we have received from the sponsors, looking at their particular porcine xenografts. On the left are the various tissues that have been examined, and we asked them to look primarily for infectious virus, since this is an endogenous virus, to look for sequences that would have been positive.

The hepatocytes have actually been tested by more

than one sponsor. This is just a compilation of the data by several different sponsors. One sponsor in particular, though, in addition to doing a culture assay also looked directly from reverse transcriptase activity. This is an enzyme that is uniquely expressed in a cell that is infected with a retrovirus.

In a number of the lots that they examined, the majority were negative for RT activity. A small minority did have what I would term borderline positive results. In this particular assay the cut-off for a positive result is two-fold above background, and in these few lots that were positive it was greater than two-fold but less than three-fold above positive.

In culture assays, though, all the hepatocytes have been negative. This is culture with human 293 cells and in some cases also with a porcine cell line called ST, and all the results from these assays have been negative.

One sponsor has also done an additional analysis. In their particular product they load hepatocytes into a device that allows for ex vivo perfusion and, since this device provides some level of a barrier they wanted to determine whether or not that would be a barrier to transmission of PERV if it were there. That analysis is negative, and the sponsor will be presenting the details of that study in a presentation later this morning.

In a culture assay with neuronal cells, again, there was no isolation of infectious virus, and one sponsor used peripheral blood mononuclear cells because their product is actually a whole liver that is used for ex vivo perfusion. This was a surrogate for that. In this case, they activate the cells with phytohemagglutinin and interleukin-2 and, again, the results were negative.

[Slide]

Now, in the published literature there are several other tissues that have been examined by culture assay, and some are in contrast to the results on the previous slide. PBMCs that were activated with phytohemagglutinin and phorbolmyristic acid were positive for expression of virus and actually did express virus that was infectious to a human cell line.

In addition, a group reported in The Lancet last year that primary cultures of porcine aortic endothelial cells also expressed a virus that is directly infectious for a human cell line.

In the case of islet cells and kidney cells, there was one report, looking just for reverse transcriptase activity directly in those primary cultures, and it was negative in the case of islet cells and positive in kidney cells. In that report they didn't correlate those findings with infectivity studies.

[Slide]

Now to move on to data regarding recipients who have been exposed to porcine cells, either ex vivo or in vivo, all sponsors to date have, at a minimum, performed analysis of peripheral blood mononuclear cells looking for pig retrovirus sequences by DNA PCR.

In addition, one sponsor has also supplemented that information with use of a Western blot that analyzes development of antibodies that would be specific against a retrovirus. The remaining sponsors are in various phases of development of serologic methods.

A third approach for looking for PERV transmission in vivo, which actually was suggested by committee members at the last meeting, was to look for plasma viremia. At this point, no sponsors have developed or incorporated that method.

[Slide]

What I was going to show on the next slide are the results first from DNA PCR analysis. This is a summary table of all the negative results that we received from sponsors by DNA PCR. On the next slide I go into a little bit more detail on the few positive results we have obtained.

Because at the time of issuance of the letter some patients in certain clinical trials had already been treated, you will see that the data is sometimes referred to

as retrospective and those patients may be several months to several years post treatment, and I call active those patients that were treated subsequent to the issuance of the letter and, so, are monitored at the time of treatment forward.

So, in the first example the product are hepatocytes which are within a barrier. Retrospective analysis of 29 patients that have been treated has all been negative, and these patients were examined anywhere from two months to five years post treatment with hepatocytes.

In the 13 patients that have been treated subsequently, 12 of those have been negative. Actually, this number is now greater. Since I made this slide last week the sponsor updated. There are additional 9 patients who have been treated that are also negative at the early time points.

For a product that uses whole liver for ex vivo perfusion, 2/4 patients who have been treated had negative results. In a retrospective analysis of 24 patients treated with fetal neural tissue all of the results have been negative, and this analysis was done on patients who had been treated anywhere from 2 months to 2 years post treatment.

On the right, you can see I just list the relative sensitivity of the DNA PCR assay in each case.

[Slide]

This is the analysis of the positive results. In the single positive in the clinical trial using hepatocytes with a barrier, this patient had a positive result that was obtained at a sample taken immediately post treatment. Subsequent samples, taken at 1, 2 and 3 months post treatment were all negative. Additional analysis of the positive sample from mitochondrial DNA using porcine specific sequences also yielded a positive result. So, the interpretation of this kind of finding is that you are most likely detecting porcine cells rather than truly an infected human cell.

In the second case of the two patients that were treated with whole liver perfusion, in the first there was a time point at six months which was initially negative. A subsequent sample at 12 months was positive, and then subsequent to that the 17-month sequel has been negative.

In the second patient there was a positive result at two weeks and one at two months time points post treatment, and a negative at 11, 17 and 20 weeks.

In both of these particular cases there was insufficient DNA to go back to test either as a repeat for the pig retrovirus sequences or to do additional analysis for mitochondrial DNA. So, it can't be ruled out that these results may represent microchimerism. The other possibility

is that at the time points when the positive results were found these samples were collected at a relatively short period of time, a similar time period from both of these patients and the samples were actually analyzed in parallel. So, at that point the sponsor was concerned that it could be a false positive due to introduction of a contaminant into the sample, and has changed the protocol and, as you can see, all subsequent samples have tested negative. So, the other possible interpretation of these results is that it is a PCR contaminant. Of course, the third possibility is that it represents a true positive.

[Slide]

This is from the published literature. There were two reports last year in The Lancet where, again, retrospective analysis was done. This was performed by the CDC in a cohort of ten patients treated with pancreatic islet-like clusters, in Sweden. These patients were anywhere from four to seven months post-transplant. The CDC used a variety of methods to analyze these patients. They did DNA PCR of peripheral blood cells. They looked for serum viremia by both RT-PCR and by PCR-based RT assay, which is a highly sensitive way to detect the viral enzyme reverse transcriptase, and by Western blot to look for antibodies. In all cases, all samples were negative. Walid Heneine will be going through this data in more detail, as well as

additional data that CDC has generated from retrospective studies.

In the second report, from Robin Weiss' laboratory, two patients who had been exposed to porcine kidneys for short-term ex vivo perfusion were also analyzed. Again, these are retrospective samples taken anywhere from six hours to three years post treatment, and by both DNA PCR of PBMCs and to look for neutralizing antibodies the results were negative.

[Slide]

So to summarize, the results that we have obtained from sponsors -- this is just another way of looking at it by exposure of the recipient, in the case where patients were exposed through short-term ex vivo perfusion and there was a barrier between the cells and the patient's fluids, in the retrospective analysis 29 patients were negative. In short-term follow-up patients, actually as I said, this number of 12 has been updated and is now 21 total patients who are now negative, and there was the one positive result which is most likely due to microchimerism.

In the second type of exposure, which again involves short-term in vivo exposure but in this case is through whole liver so there is no barrier -- there is a mistake on this slide, there are actually 2 negative patients and 2 positive patients.

In the third type of exposure which involves in vivo direct implantation of cells, we have long-term survival of the cells and there is no barrier between the porcine and human cells, 34 patients examined retrospectively have all been negative.

[Slide]

Now, I want to again reiterate that this is a summary of data we have received based on DNA PCR analysis, and I just want the committee and the members of the audience to be aware that there are some inherent problems with DNA PCR analysis of PBMC. For example, this type of analysis when you have a low-level infection, something that might look like microchimerism could, in fact, be a low-level infection.

Secondly, the inherent rate of false-positive results in PCR is another problem that needs to be overcome. Third, there is data suggesting that human PBMC may not be a natural target for infection. So, this may not be a reasonable place to look.

[Slide]

I wanted to just quickly go through some data that we at CBER have generated, where we have tried to infect human PBMC in vitro and we were unsuccessful. So, we actually looked at a variety of hematopoietic cell lines to determine whether or not there was some lineage that we were

missing in our in vitro culture conditions that was more susceptible than others. As you can see, the cell lines that are highlighted in blue were the ones that did become productively infected, and the results from that data is shown in the bottom half of the slide.

[Slide]

But we were able to infect cell lines representing T cell, B cell and myeloid lineages. We then used these cell lines that became positive as a producer virus, with the idea that perhaps these may be better adapted to growing in hematopoietic cells, and tried again to infect PHA-blasts of human PBMCs and, after an 8-week culture period, they remained negative by RT-PCR. As a positive control we were able to infect 293 cells and we know the cells lines were productively infected. So, this is just a note of caution that PBMC, at least in vitro, don't appear to be susceptible to infection.

[Slide]

In the last few minutes of my talk I wanted to quickly go through some data that we have generated at CBER, looking at porcine plasma-derived Factor VIII -- plasma-derived product. This, actually, is something that we have done in conjunction with CDC, and it was brought to our attention by CDC when they contacted us about a year ago to say that they were looking at lots of this particular

product, called hyate C, and found that there was PERV RNA present when they looked by RT-PCR.

This particular product is used in hemophiliac patients who develop inhibitors to the human Factor VIII. So, it is used in a small minority of hemophiliac patients and it is really a treatment of last resort for these patients. So, at that time we didn't want to prematurely take this product off the market. We certainly notified the Hemophiliac Society that there was this data but we felt that it was really imperative to do additional analysis. So, we did confirmatory testing of CDC lots and did additional testing to look at whether or not these results correlate with infectious virus present in this product.

So, of five lots that CBER received from CDC, we did confirmatory testing and we, like the CDC, also found that they were positive for pig retrovirus RNA. We also used a PCR-based reverse transcriptase activity assay. Keith Peden and Tom Maudru, from the Office of Vaccines, did these assays for us and they also found that they were positive.

[Slide]

So, to determine whether or not the product also contains infectious virus we took six lots of hyate C, and we started with lyophilized vials that had been stored at minus 70. So, I wanted to emphasize that these had never gone through a round of freeze/thaw which may limit your

ability to detect infectious virus.

We directly re-suspended them according to the manufacturer's instructions and then inoculated them into three different cell substrates, human 293 cells, feline PF-4 and porcine ST cells. These are three cell substrates that have been shown to be susceptible to infection by PERV. As a positive control we included dilutions of PERV to look at the sensitivity of these three cell lines. The human 293 and the feline cell lines could detect out to 1:1000 dilution, although ST at 1:100 became positive. We also did direct inoculation of hyate C with PERV to control for a potential inhibition of detection of virus if it were present. Then we took these same six lots and directly analyzed them for viral RNA and for reverse transcriptase activity.

[Slide]

These results are shown here. These are the results of the PCR-based RT assay performed by Tom Maudru and Keith Peden. On the Y axis are the units of activity in pico units porcine endogenous retrovirus microliter. The blue bar on the right represents tissue culture fluid from one of our cell lines that is productively infected. So, it is a positive control. All the lots tested, which I have just randomly labeled A through F are positive. They all have detectable levels of RT activity in this assay. As you can see, they are all 4-5 logs lower than our tissue culture

fluid.

[Slide]

This was our positive control where we spiked hyate C into the lots. The set of bars labeled "none" are cells that were directly inoculated with PERV without hyate C and A through F again are lots of hyate C that were inoculated with PERV.

As you can see, we were able to recover virus in all of those with the exception of lot D, where we were able to get positive results but there was some significant inhibition in that particular lot.

[Slide]

We carried the cultures of the hyate C without PERV in them for a total of 8-9 weeks. We analyzed these cultures every 2 weeks for viral RNA expression by RT-PCR and in all cases they were negative. At the end of the culture period, cellular DNA was analyzed by DNA PCR and, again, these were negative.

[Slide]

So, we concluded from these studies that although there is viral RNA and viral reverse transcriptase present in final product, there is no evidence for infectious virus in the final product and that, most likely, the positive results for RNA and RT activity represents inactivated virus that is not removed during manufacturing.

[Slide]

So, I just wanted to finish with a quick summary of what I have told you this morning. First, analysis of various porcine tissues, both by sponsors and what has been published in the literature, shows that expression of infectious virus is tissue specific. Not all porcine tissues express infectious virus, and that most likely also the differentiated or activated state of the cells may impact whether or not infectious virus is present. These results aren't surprising. It is quite similar to what is seen, for example, in the murine endogenous retrovirus system.

Looking at transmission of PERV in recipients, to date most of the data that we have seen from sponsors has relied on DNA PCR analysis of PBMCs. Most of this data has been negative. The very few positives that we have seen are most likely due to either microchimerism or problems of a false positive or contamination in the PCR reaction.

I showed you data suggesting that human PBMCs, at least in vitro, are not susceptible to PERV infection. We don't know whether or not that translates to the in vivo situation, but it certainly highlights a need for developing other methods to analyze patient samples.

Finally, in the porcine Factor VIII story, although we were able to detect viral RNA enzyme in this product, there was no evidence for infectious virus in this

particular product.

I am going to stop there. I think we are waiting to have questions at the end of all the speakers. Correct? And, I saw John Coffin come in during my talk, and I wanted to welcome Dr. Coffin. He is a member of this subcommittee and he was a co-organizer of this year's Banbury Conference on Xenotransplantation. He has kindly agreed to provide a summary to you today of the findings of that meeting in order to bring to bear any emerging scientific data. Dr. Coffin?

Xenotransplantation: A Scientific Basis for Risk Assessment

DR. COFFIN: Thank you very much, and I apologize for my late arrival. I am not quite yet used to the 270 traffic.

[Slide]

Last month, Robin Weiss and I organized the meeting at the Banbury Center, on Long Island, to discuss what you see here -- Xenotransplantation: A Scientific Basis for Risk Assessment. This meeting was organized partly as part of the Hastings Center, which is a bioethical think tank in New York. The meeting, although small, ranged over a number of different areas, including applications and challenges of xenotransplantation; a lot of talk on ethical issues; discussion of regulatory and policy issues; and, finally, discussion of infections, particularly viral,

disease risk associated or potentially associated with xenotransplantation technology.

My discussion today will be limited only to the last of these points because that is certainly all that I feel qualified to discuss for sure. I want to make several other caveats. This was a very small focus workshop. Many people with interest in this field could, of necessity, not be present. So, this is not by any means a comprehensive report but an update simply of what was discussed at this meeting. A lot of what was discussed is in the nature of review, and a lot of what I will present is in that nature. A portion of it will overlap what you have just heard from Carolyn, who was at the meeting, and a fair amount of the data she presented here was presented there. Finally, quite a number of the people whom I am going to quote, to my dismay, are in the audience or on the board and, so, if I misquote you, you can come up to me quietly afterwards and pat me on the shoulder, or if I over-quote you or anything else I will take some measure of responsibility for doing that.

[Slide]

So, the infectious disease risk -- as I said, we focused really pretty much only on viral diseases but we can break it into two different types, first, exogenous viruses and by that I mean agents which are not transmitted

genetically or transmitted horizontally from one animal to another or vertically during birth but which do not form part of the genome and, secondly, and as you have already heard discussed from Carolyn and the reason for the greatest concern at this point, endogenous retroviruses, particularly the porcine endogenous retroviruses or PERVs, also called PoEV and PoERV. This is my particular preference but I am not going to impose that.

Several different areas were discussed -- biology and distribution, testing for infection, and Carolyn just gave you an overview of that, as well as risk assessment of these viruses. So, these are the areas that I plan to share with you in the next few minutes.

[Slide]

First, there were several examples of exogenous virus infections that might be of some interest to think about that were presented at the meeting. I will say here that I am not going to go talk by talk; I will jump around from one speaker to another but I will put attributions as we go along. Parrish discussed the rather striking situation of the origin of the canine parvovirus, which was originally a cat only virus, and apparently a few mutations, somewhere in the vicinity of 1976, or at least first detected in 1976, occurred, and within a span of a very few years this virus had spread worldwide, even into rather remote wild animal

populations. There has been a series of subsequent revolutionary events that have virtually replaced this virus. So, the potential for sort of mutant viruses with extended host range, given the probability of viruses like this to be deposited in feces and carried onto airplanes and then spread around the world, which is the likely mode by which this particular virus has been spread, is certainly very well documented in this case.

The second kind of example, and this is also an example of a virus which with a relatively small change in its genome, a series of small changes, has changed its host range so that it can spread to completely different animals and then, in fact, can subsequently change and spread back again into the original cat population.

In the case of arenaviruses, which is a family of viruses found worldwide, particularly in rodent populations, many of which are benign -- this was discussed by Mike Buchmeier -- despite very high titers, some of which, for example Lassa, are high virulent in humans but don't in general spread to a great extent from one human to another. Also viruses in that group are hemorrhagic fever and hanta viruses of the New World.

Hanta viruses is a particularly interesting case. They are prevalent in many parts of the United States, particularly in the north east and, in fact, in Baltimore a

study showed that there is a 5 percent total incidence of this virus in people, and this tends to be particularly in households with infected mice. So, there is a lot of ongoing introduction of this particular virus into the human population but never taking hold.

[Slide]

A case that wasn't discussed but I thought I would throw on here because it is of particular interest I think, and actually I am sort of sorry we didn't have somebody presenting it at the meeting, is J ALV, which is a recent avian leukosis virus that only has shown up really in the last few years. Avian leukosis virus is widespread in commercial bird populations causing a certain amount of mortality but under relative control. However, there are endogenous proviruses in chickens related to these, some of which have apparently been in the bird population for quite a long time in the genome, and are quite old and are thought to be pretty much completely defective but sometime, probably not too long ago, there was a recombinant combination of that between this exogenous virus and one of these old ones which provided a new envelope gene, therefore, a new host range. This virus probably spreads both vertically and by vaccination where one takes the same equipment and does thousands or hundreds of thousands of chickens. This virus has spread throughout particularly the

broiler industry and has caused hundreds of millions of dollars worth of damage in the last few years. So, there may be an object lesson here that might be worth keeping in mind.

David Onions talked about large numbers of porcine viruses, 70 or 80 I believe that are known, and discussed the ones that for xenotransplant should be of specific, special concern, for example, viruses which cause known zoonotic infections and can go from pigs to humans; viruses that, although not known to cause such infections, can replicate human cells; viruses that can undergo abortive infection and, therefore, are potentially oncogenic; or viruses that are known to change host range or potentially able to do so; and also viruses that cannot be easily eliminated by hysterotomy; and barrier breeding such as parvoviruses and circoviruses and CMV, and these are all of particular concern.

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I am going to talk about measures that they are discussing for derivation of clean animals. It seems impractical to maintain animals, like mice for example, under completely germ-free conditions but deriving a breeding stock under conditions where there is the least possibility for the passage of infectious agents and maintenance under conditions that are as clean as absolutely

possible, including health screening of staff so that infection isn't brought in by the people working with the animals, including subtleties such as recent vaccinations may well have the risk of bringing in viruses that could infect the animals and subsequently, of course, be passed on; and then close monitoring.

A point that can be taken about many of the viruses that are of concern is that we do not seem to have yet adequate tests for this purpose, good quantitative either serology or PCR or other tests. So, there is a considerable amount of development work required to ensure that this exogenous virus problem is as well contained as one thinks it should be.

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A couple of anecdotes -- well, not anecdotes but small number of examples from some recent studies involving baboon transplants where Marian Michaels looked for baboon cytomegalovirus in several transplants, one of which was transiently positive but subsequently disappeared, and Jonathan Allan talked about a couple of studies looking at baboon foamy virus where he could find some DNA, no seropositivity and the DNA was probably due to microchimerism although, as was pointed out in the previous talk, it is a very difficult issue to deal with, very hard to sort out in the case of where one is looking for DNA to

distinguish virus in infected cells from virus that has newly infected recipient cells.

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There was a fair amount of concern expressed in the meeting that perhaps disproportionate attention is being paid to endogenous virus issues and one should look more closely at the exogenous infection issue.

It was also noted that there is considerable challenge in detecting and controlling these viruses; that new viruses are appearing on the scene. There has been a new porcine virus that is also fatal for humans. It appeared in Malaysia a few months ago, and this continues to happen and constant vigilance is required for this.

But all in all, I think the feeling -- at least the feeling I came away with was that with proper controls risk of infection of exogenous passage viruses are likely to be less than the very severe risk of infection of the currently used allotransplant technology and, therefore, at least from the standpoint of exogenous viruses the benefit, given proper controls, of xenotransplantation is likely to be quite high.

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Then, of course, there was considerable discussion of endogenous viruses, particularly the porcine endogenous viruses. I will just give a little bit of background before

I go into this. Endogenous viruses are retroviruses that have found their way in a DNA form into the germ line. They are found in all vertebrate species and humans carry thousands of these. We all probably have pretty much the same complement of them since, as far as we know, all the viruses in humans were introduced some number of millions of years ago and I think we are pretty much genetically homogeneous in that respect. That is not true of many other species, including pigs, where the viruses were introduced into the germ line relatively recently in evolutionary terms and, in some cases, some of these proviruses -- there are in the group we are talking about perhaps 30 or so per animal, depending on the strain. Some of these can clearly yield infectious virus. You just heard Carolyn talking about some particular cases of that.

In cats, just as an example discussed by Steve O'Brien, there is the virus RD114. RD114 is an interesting story for those of us who were around back in the early '70s. It was found as a virus that was produced by human tumor lines that had been originally generated by passage through kittens. It was believed, and a press conference was held -- of course, this was the early days of the Special Virus Cancer Program -- this was believed originally to be human retrovirus and was very rapidly shown not to be, but to be a xenotropic virus of cats, that is, a virus that

could not infect cat cells efficiently but could infection human cells and was readily transferred into these cells and infected these cells in the xenotransplantation, if you like, of a human tumor in kittens.

A very similar phenomenon was seen in mice where I, as chair of the IVC over the years, have had a number of people walk into my office with human tumor lines that were pouring out of really lovely C-type retroviruses and usually the first question I asked these people was were these tumors ever passed through mice. Human tumors passed through mice have a very high probability of having been infected by an endogenous mouse virus and then subsequently expressing that virus. In fact, there are some commonly used cell lines around that have that property, some of which may be being used for genetic engineering purposes.

This group of viruses is present only in the genus to which domestic cats belong but no other groups, but it is related to endogenous virus in baboons which implies that there was some cross-species transmission event, perhaps from baboons to cats but there may have been some intermediates, and so on, some two million years ago.

So, this gives you a feeling for how these viruses come in and can be passed on in the germ line only to reappear at rather awkward moments later on when you present the opportunity in this particular coculture for having

viruses of humans and cats together. I should also point out that there has never been any evidence of infection in humans with this virus by handling of cats or, so far as I know, in veterinary surgical procedures. So, it doesn't mean this virus, once it got in, could be spread from person to person but you certainly create an opportunity here for infection that may not otherwise occur in natural circumstances.

[Slide]

Porcine endogenous virus is a murine leukemia related endogenous virus. I believe this group is now called gamma retroviruses, found in multiple copies in all pigs examined, and related sequences can be found in all *suidae*, in all pig-related species. Infectious virus is released by most porcine cell lines, except for the ST line which was mentioned before, and this, as Carolyn was discussing, is therefore a useful cell line for testing infectivity of these viruses and it can be released, as you have already heard, as activated lymphocytes.

Three principal subgroups have been identified by sequence testing of the envelope gene which includes both sequencing host range and interference testing. These are called A, B and C. A and B can detect human cell lines reasonably well. C, which actually I believe is not really identified as a virus but only as an envelope gene, can

affect almost only pig cells, with the exception of one human cell line. So, these two viruses, in the rather confusing terminology that was originated with mouse retroviruses, could be referred to as polytrophic viruses. It should be pointed out that just because they have the same host range doesn't mean they use the same receptor for infection. Interferon studies indicate that these two viruses actually use different receptors.

Multiple copies of these proviruses are found in all pigs tested. C is found in high copy number in mini pigs and either less or not at all in other strains, and there is considerable polymorphism from one individual to another.

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This is my memory of an unrooted phylogenetic tree relating these envelope genes. So, it gives you the feeling actually that as you sequence you can detect more and more of these, like C_1, B_1 and A_1, which are not necessarily known to belong to infectious proviruses but which relate to the known infectious endogenous sequences, the As, Bs and Cs, and as one sequences this thing will certainly bush out more and more as we go along, and there may well be more subgroups hiding in there. A and B are the main ones that are seen as being produced by the cell lines that I mentioned.

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As Carolyn pointed out and as I think she discussed earlier, virus isolated by the induction of lymphocytes can adapt in some way to human cells, either by getting more infectious or by segregating out less infectious virus from the population.

The most efficient isolation actually starts -- at least some cell types require starting with cocultivation with pig cells and then passage through human cells, raising the possibility that in many cases there may be some additional either pseudotyping by, let's say bringing in additional envelope genes, or actually combination events where the pig cells between pig viruses may actually be giving rise to the most infectious virus types for humans. These viruses are not only present in pigs as endogenous sequences, David Onions showed that you can detect low-level viremia in at least some pigs.

As I said, there are a large number of proviruses, at least 30 or so per animal, many of which are different from one animal to another, that is to say, they are integrated at different sites. So, they are different coviruses probably with some different biological properties. Many of those, probably most of those based on our experience with other endogenous retroviruses, are likely to be defective in one way or another.

At least six proviruses are common to all pigs and, therefore, could not be bred out if one were to start on an ambitious breeding program to remove proviruses at different chromosomal sites. Whether these represent infective ones we don't know yet.

In addition to the C type or gamma retroviruses, if you like, if one starts looking, as with all other species that we know of, at least all other mammalian species and virtually perhaps all other animal species, if you go looking you can find other types of endogenous proviruses. For example, PCR probes that were general for reverse transcriptase genes and some viruses turned up that resembled groups of endogenous viruses that are found in large numbers in humans and other types of species, D type or also related to mammary tumor viruses. Whether these represent infectious elements as they would in some monkey species or non-infectious viruses which would be the case in humans, remains to be determined but we have probably only scratched the surface in terms of endogenous provirus-like elements in pigs or many other species.

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There was a certain amount of discussion of the testing for these in animals and in human recipients. Potential tests include serology. For example Western blot for the p30 gag kapsa protein has been developed; use of RT-

PCR plasma, PCR in lymphocytes and amplified RT assays, a very sensitive assay for RT-containing virions.

Carolyn discussed the issue of mitochimerism and the difficulty of detecting that, and that still remains an issue and there was actually a fair amount of discussion on the problems of separating out whether you are detecting an infected human cell or an infected pig cell.

Most testing of patient samples to date is negative. I think we are going to hear a lot more about that in this meeting, including some patients who have received extracorporeal perfusion or allocell transplants. There are a number of monkeys that had heart or kidney transplants, and a large series of patients and others exposed to pig products, although there are some hints that perhaps there may be one Western blot positive individual. Maybe we will hear more about that.

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Finally, as I think everybody should be aware and is one of the reasons we are having meetings is that the real risks for this infection are unknown. In order for a PERV transmission to actually result in an event of concern a number of steps have to occur -- spreading infection of the recipient from an initial production of virus infecting a few cells of an individual, spreading infection has to occur. The event has to lead, at least in some individuals,

to pathogenesis. If it didn't, it perhaps wouldn't be of great interest although it would certainly be of some concern. Finally, transmission from one individual to another -- if the virus only gave rise to the sort of pathogenesis one usually sees with this type of retrovirus where a fraction of individual recipients had some sort of malignancy at some very long time after the event, this might well be an acceptable risk in cases where the initial event was life-threatening and there was no choice. The risk or the concern of what happens if a transmissible virus is created is uncovered or created by this step is obviously much more serious. The Stoye scale was brought up where each of these events is considered to be progressing from likely to unlikely to very unlikely to very, very unlikely, with some sort of undefinable scale attachment as you go along.

I think there was a general feeling at the meeting, and perhaps that is something that will be discussed again, that it would be very valuable to develop some kind of animal model for PERV infection so that, should there be some problems -- at least as far as was discussed at that meeting, there wasn't a feeling that the progression of the development of transplant technology should be held up waiting for this model, but that this should be a parallel track and that it would be important, should there be evidence for infection and so on, to have some sort of

model to be working from even if it wasn't considered to be the optimal model.

I think with that I will close and thank very much Carolyn Wilson and Jonathan Stoye for helping put this presentation together. Thank you very much.

DR. SALOMON: Thank you very much, John and Carolyn. I am actually doing very well on time, and I ask for the chairman's prerogative to ask some questions at this point which are not traditionally on the agenda. We do very much want to stay to time so that gives us about five or so minutes that we could open up for some questions.

DR. VANDERPOOL: I have one question that is more common sensical and it certainly may be important for the public members who are here, and I would appreciate it, John, if you or Carolyn could comment on what you mean by infectivity. It is really on your second to the end slide. You distinguish between infectivity, pathogenesis and transmission. But I think for commoners who are non-specialists infectivity sounds really serious. It sounds like you have a disease and you can pass it on. Could you, for the sake of the public and other persons present, make some of those distinctions a little finer for us?

DR. COFFIN: I should preface by saying that we and other animals get infected with many agents that we actually never recognize the infection of. So, infectivity

and disease induction are two very different things, and that is particularly true in retroviruses where there are many retroviruses, including the relatives of HIV in monkeys actually which can infect individuals and never give rise to significant disease.

So, it is a very important distinction, and there are two levels of infectivity that are important in this particular case. One is at the local site where there are cells of the donor, the pig for example, immediately adjacent to human cells. Whether some virus gets transmitted to those cells immediately adjacent to the first step, and so first infection. Then, the second step is whether those cells can pass virus on to the rest of the body.

So, there are two very different issues here, the issue of whether you can locally infect a few cells and whether then that goes on to create a spreading of infection that can actually spread through the whole body, very large numbers of cells in the body and create much, much greater opportunity for any kind of disease effect, if that is going to happen.

The infection of a few cells I think is probably, given the models we have to work with, considered to be not an unlikely event. Widespread infection is probably considered at this point to be much more likely but, again, we don't know what the scale is. Infection of a few local

cells is much harder to distinguish than widespread infection would be, leading to viremia, virus in the blood, infection of cells at different system locations.

DR. HIRSCH: I have a question for Carolyn. I think you importantly suggested that human PBMCs might not be infectable with PERVs and, yet, a lot of requests are being made to test human PBMCs. My question is have you done investigations of subsets, like CD4 cells, CD8 cells, monocyte macrophages within PBMCs because, as you know, in other systems like HIV sometimes the CD4 cells become more infectable when you take the CD8 cells out.

DR. WILSON: No. That is a good point, we haven't tried those manipulations. Instead, we tried going back to just cell lines and lineages to first get an idea if what certain lineages might be more susceptible, but that is a point well taken.

DR. ONIONS: Carolyn Wilson very elegantly showed the difficulty of infecting human peripheral blood mononuclear cells. The caution I have is that we know that certainly in animal models we find widespread action of peripheral blood mononuclear cells that are very difficult to infect in vitro, just as human cells are with PERVs but the root of infection has to be through a stem cell. So we should be cautious in dismissing using PBMCs as a target just on the basis that they are quite difficult to detect in

vitro.

DR. SALOMON: Yes, Dr. Allan?

DR. ALLAN: I have a question for Carolyn, and this is probably something that we can discuss during the meeting, which is the question of contamination with PCR because when you get a positive result, obviously, the issue is whether it is contamination or not and if we get positive at one time point and negative at another time point do you dismiss it as contamination? How do you address the issue of contamination? Do you do multiple samples? Because if you put it into the realm of contamination then you are sort of dismissing -- you may be dismissing all PCR results and why do anything? I mean, it is really difficult.

DR. WILSON: Well, that is why I didn't eliminate the possibility that it wasn't a true positive result. I don't think the data at this point -- because there weren't enough samples to go back and do repeat testing, it can't be eliminated as a possibility.

DR. ALLAN: Because you could have instances, especially in microchimerism, where you get a positive at one time point and you get a negative at another time point.

DR. WILSON: Yes.

DR. SALOMON: Dr. Michaels and then Dr. Mickelson.

DR. MICHAELS: Another question for Carolyn, I don't know if you have this data; it might be something that

we will need to bring up with some of the sponsors later but, again, bringing up some of the limitations which you have shown with the PBMCs, have any of the sponsors looked at other tissue samples of, for example, some of the patients who had the hepatocyte barriers if they then later underwent an allotransplantation for the liver is the native liver available that could be tested, or has it been tested?

DR. WILSON: Nobody has done those types of experiments.

DR. SALOMON: Dr. Mickelson?

DR. MICKELSON: Just a general question maybe for Carolyn and other experts on the panel, a lot of the assays seem to depend on reverse transcriptase PCR which, in the context of the patient, means if you can pass it you are already looking at a situation where infection has been established, which is unfortunate. Is there some other way or some other assay that could be used to give you a hint that something might be happening before there is established infection in the patient? Can you look for the appearance of antibodies in patients against PERVs or something? It seems that if you have an analysis that only gives you a positive once something has happened, maybe if you had an earlier step assay that could be done -- I would like some discussion of that.

DR. SALOMON: Yes, specifically to that I would

say for the record it was a recommendation that was, I believe, accepted by the FDA from this board the last time we met that we should put an emphasis on developing serological assays parallel to PCR technology based assays.

DR. MICKELSON: I just didn't see that in some of the information that was here, but just the earlier you can move on into the event the better it is for patients.

DR. SALOMON: There was mention in Dr. Coffin's slides of a Western blot study. John, do you want to comment on that?

DR. COFFIN: Well, I will ask David to comment on it. He is the one who has been developing it.

DR. ONIONS: Well, I can comment on the serology in two senses. One is that we have been using recombinant p30 and we are now putting a recombinant ending to screen patients for antibody. We have looked at well over 200; the 300 on John's slide includes controls and validation. We know that the assay doesn't pick up antibody to HIV and HTLV so that we are confident of its specificity. We do have patients that do have antibody. We have confirmed that there is antibody binding by using mass spectrometry techniques. This is actually probably bound to p30. So we are confident there is antibody to p30. What we don't know is whether that epitope could be due to cross-reactivity. So, we can't dismiss the possibility that this is quite spurious. If you

screen people for HTLV capsid antibodies, you will find families that appear to have antibodies to that and, yet, there is no other evidence that they are HTLV infected. So, we need some caution there.

But just in terms of using serology, I think it is an important adjunct in testing but my caveat is that, again, this is not HIV. In the gamma retroviruses normally antibody is associated with a recovery phase rather than with concurrent active infection. Usually it is a secondary event. So, I am not sure it is useful in a diagnostic sense in the human that it is a very early indication rather, in my view, it is an indicator of exposure and often of recovery. So, it is a rather different indicator.

DR. MICKELSON: Then, what would you suggest as an indication for early stage?

DR. ONIONS: Well, we know that the earliest phase that we see in animal models and human models where you take amphytropic virus and put it into primates, or you look at cats, that the first thing that you see is a transient infection where you see virus in the plasma at a low level. You then get infection, usually in the bone marrow, and then you get a higher titer plasma viremia and also virus in cells. So, using, as John hinted, developing techniques to look for virus in the plasma is an important adjunct in testing, and one that I would encourage.

DR. SALOMON: I am afraid, just to stay on time, we are going to have to go forward. This brings us to the second phase of this morning in which we have invited some guests for presentations, and the first presentation is from Dr. Gillian Langford, from Imutran, entitled, In Vivo Expression of Porcine Endogenous Retrovirus in Pigs Analysis of Non-Human Primates Transplanted with Porcine Organs for Evidence of Cross-Species Transmission of PERV.

Guest Presentations

In Vivo Expression of Porcine Endogenous Retrovirus in Pigs Analysis of Non-Human Primates Transplanted with Porcine Organs for Evidence of Cross-Species Transmission of PERV

DR. LANGFORD: Thanks.

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I want to talk about two studies that we have done at Imutran and in collaboration with GTI which is a company based here, in Washington, primarily to look at expression of endogenous retroviruses in the pigs that we are planning to, or hope to use in clinical transplantation, and a study that we have done where we have analyzed non-human primates that have been transplanted with organs from pigs to look to see if those primates have been infected by the virus.

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The studies that we have done to look for expression of the virus have really been performed to

supplement the data from the in vitro studies. We have analyzed expression in out pigs using three different techniques, the Northern blot analysis, transmission electron microscopy and an assay that looks for an enzyme that is associated with the virus.

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We have done Northern blot analysis on families and non-related pigs, from about ten different pigs within our herd of animals, and what we have done is both Northern blot and RT PCR analysis and we have taken a variety of tissues from our pigs to see whether we could see viral mRNA expression.

What we have found is that when we look in a variety of tissues -- the heart, lung, liver, kidney and spleen -- we see viral expression.

We have also established an in situ hybridization technique which we have used to look in neural cells from our pigs, and we have found that by in situ we can see viral expression of our mRNA expression as well.

Interestingly, what we have found is that the highest level of expression, and this is from tissue to tissue within our pigs, is within the lung, and this is consistent between all the pigs that we have looked at. We see high levels of expression in the lung and much lower levels of expression in the kidney and heart tissues.

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As well as looking for mRNA expression, we wanted to look to see if we could see viruses actually being released from our pig tissues, and we did this by transmission electron microscopy. What we did, we took a series of tissues from our pigs and analyzed them by TEM. In addition, from some of the primates that have been transplanted with the pig organs we took a variety of tissues from those primates and also analyzed those by TEM.

The results that we found when we looked in the tissues from our pigs were that there was no evidence that we could see any viral-like particles when we looked in the heart, kidney and bone marrow. In addition, from our primates when we looked in the spleen and lymph nodes, again, we couldn't see any evidence of virions being produced.

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However, when we took serum from our pigs we actually saw evidence of viral-like particles by TEM analysis, although when we did the immunogold analysis these TEM particles didn't bind antibodies against them, the recombinant gag, or antibodies directed against the whole virus.

However, because we had seen these TEM results of these viral-like particles in porcine serum we went ahead

and analyzed porcine serum using the product-enhanced reverse transcriptase. This is a two-stage assay. When exogenous RNA is added to a test sample, if the test sample contains reverse transcriptase the RNA is converted to cDNA, and then that cDNA is amplified. So, if you get a positive signal in your PERT assay, this indicates the sample contained reverse transcriptase.

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As I said, what we did was to take a whole series of serum samples from our transgenic pigs. We fractionated those serum samples through a sucrose gradient to ensure that we were really looking at viral particles, and then we analyzed those samples using the PERT assay. What we found is that we had positive results, indicating that we may have a low-level viremia in our pigs.

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So, what we wanted to do was really to assess whether that PERT activity was associated with infectious virions. So, we did this using infectivity studies in which we cultured human cell lines which were known to be permissive for PERV. In this example we used human 293 cells in the presence of porcine serum for up to ten passages. After this, we harvested the human cells and analyzed them by PCR to see if there was any evidence of infection.

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What we found is that when we looked at these human 293 cells there was no evidence of infection. So, that led us to conclude that although we can see PERV activity in our pig serum, this isn't necessarily associated with infectious virions.

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As I said at the beginning, not only did we look for viral expression in our pigs, we have also analyzed samples from both baboon and cynomolgus monkeys that have been transplanted with transgenic pig organs to look for evidence of infection in these primates. At the moment there is some debate about the validity of using primates to look for cross-species transmission of the virus, primarily because there is very little in vitro data that shows that primates are permissive or the cell lines are susceptible to infection by PERV.

We have actually generated some in vitro data that shows that both rhesus and chimpanzee cell lines are actually susceptible to infection by PERV, and we think that supports the model that we have used.

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So, basically, the model that we have employed has involved taking samples from baboons or cynomolgus monkeys that have been transplanted or orthotopically with transgenic hearts and kidneys. All the primates that we

analyzed samples from were actually immunosuppressed, and the survival times of the primates that we took tissues from were between 9-99 days.

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As well as taking terminal necropsy samples from our primates and analyzing those by PCR and Western blot analysis, we are also collecting serum samples from our primates so that we can analyze them by RT-PCR to see if, when we transplant the porcine organ, it stimulates the release of additional virions into the primate serum.

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This slide describes the testing strategy that we have used to analyze the tissues that we have collected from our primates by PCR. We start the analysis by doing a PCR based on the pol gene of the virus. If the result from this PCR analysis is negative we can conclude that the sample hasn't been infected. However, if we get a positive result we don't know if that result was due to microchimerism or natural infection of the primate tissue that we are looking at.

So, to distinguish between microchimerism and infection we have developed a second PCR assay based on centromeric repeat sequences that are present in all pig cells. We know that there are approximately 2.5 thousand copies of this repeat centromeric sequence so we know that

we have developed a highly sensitive assay to detect pig cells in our primates.

If when we do this PCR assay we don't detect any centromeric sequences, then we can conclude, because we have a positive PCR result for the virus, that the primate has been infected. However, if we get a positive result for the centromeric sequence, then we are in the position where we have to resolve between microchimerism and infection.

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We have tried to resolve between these two possibilities of microchimerism and infection by developing quantitative PCR assays. What these assays involve is determining the number of copies of PERV sequences in the sample and the number of copies of centromeric sequences. When we have those two values, we then determine the ratio of PERV to centromeric sequences.

What we know from analyzing a variety of our pigs is that we know the ratio of PERV to centromeric in our donor pigs. So, when we look in our primate sample, we can then compare that ratio to the ratio that we find in the pig cell. These are just examples. If you have microchimerism, this ratio of PERV to centromeric sequences would be the same as you would find in a pig cell. Whereas, if you had an infection this ratio would be altered. You would expect to see a higher number of PERV sequences relative to the number

of centromeric sequences. So, if this ratio is altered we can conclude that the tissue that we are looking at has actually been infected.

However, there is a caveat to this testing approach that we have used. If you have a very low-level infection in the presence of microchimerism the value of PERV sequences that you see would only increase a fraction, and statistically you wouldn't be able to distinguish between this ratio and the ratio that you would see for microchimerism. So, it is possible that if you have microchimerism and a low-level infection you really wouldn't be able to discriminate between those two possibilities.

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This slide shows some of the work that we have done to validate the PCR assays that we have used, and this work is really all being done at GTI. This graph shows that when we use between 10 to 10^6 copies of plasmid containing the viral sequences that there is a linear relationship between the number of copies of the virus and the threshold signal at which you would consider the sample to be positive.

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We know that by adding spiked or by adding plasmid containing the virus to a background of human cells that we have been able to determine sensitivity of our assay, and we

know that if we spike in one copy of the virus to 500,000 human cells we can actually detect that virus. However, because of Poisson distribution we actually claim that the limit of detection of our assay is ten copies of virus per 500,000 cells.

The centromeric assay that we have detected has a slightly different sensitivity than the PERV PCR assay that we have detected. Here we can detect 10 copies of the centromeric sequence porcine endogenous retrovirus 500,000 cells.

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As well as analyzing the primate samples by PCR, we have also taken the terminal serum samples and analyzed them by Western blot analysis to see if there have been any antibody responses. The Western blot assay that we have used involves using either recombinant gag protein or whole PERV virus isolated from infected human cells. As a positive control we have antibodies that have been taken from rabbits that have been immunized with either recombinant p30 gag protein or the whole virus.

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So what are the results that we have seen from the analysis of our primate samples? What we have done is taken a whole series of tissues from our primates. Predominantly we have looked in spleen and lymph node samples, and we

chose those samples initially based on the premise that if we expected to see microchimerism in allotransplantation, they would be the tissues that you may expect to see it in. But in addition to looking at spleen and lymph node samples, we have also looked in heart, lung, liver, kidney and testes samples from our primates.

What we found is that when we look at the ratios of PERV to centromeric sequences in all the tissues that we have analyzed, and that is a total of 100 tissues so far, we don't detect any evidence of infection in those tissues. However, what we did find, and what we were most surprised to find is that in the majority of the tissues that we analyzed there was evidence of pig cells. We found both PERV and centromeric sequences.

What we don't know at the moment is whether these cells are really indicative of true microchimerism or they just released from the pig cells during the rejection process.

[Slide]

As well as the PCR results, we have Western blot results from 40 of the primates that have been transplanted with transgenic organs, and in all these Western blots we haven't detected any evidence of anti-PERV antibodies.

[Slide]

So, in conclusion, although we have seen PERV

activity in the serum from our transgenic pigs, the in vitro coculture experiments we have done, where we have passaged human cells in the presence of porcine serum, suggest that we don't have any infectious virions present in our pigs.

In addition, when we have analyzed primates by both PCR and Western blot analysis we haven't had any evidence of infection in these primates, and that is, we believe, now supported by our in vitro coculture results.

[Slide]

Finally, I would just really like to acknowledge the people who were involved in these studies. Thank you.

DR. SALOMON: Thank you very much for staying on time. I think it is obvious to everyone that Dr. Auchincloss has joined me, to my right, and with that I will yield the chair to him.

DR. AUCHINCLOSS: Dan, thanks very much for filling in, and I apologize to the FDA and my committee members for being late. I believe we are moving on now to Zorina Pitkin, Circe Biomedical.

HepatAssist Liver Support System Containing Porcine Hepatocytes

Case Study: Bioreactor PERV Analysis and Risk Assessment

DR. PITKIN: Good morning, everyone.

[Slide]

This presentation concerns in vitro PERV analysis

and risk assessment of the HepatAssist liver support system containing porcine hepatocytes. There are three parts in this presentation. First, we will introduce the HepatAssist system and its specifics. Then, we will have a brief overview of in vitro and in vivo PERV studies. Finally, we will present a case study on in vitro PERV analysis of the HepatAssist system.

[Slide]

To start with the introduction of this system, the system has been designed to support patients with acute liver failure until the liver regeneration or organ transplantation. As an update, we have conducted a Phase I/II clinical study to assess safety and tolerability of the system in patients with acute liver failure indication. It was completed in 1997. We are in the process of conducting a Phase II/III multicenter, randomized clinical trial that was initiated in 1998.

[Slide]

On the right-hand side you see the picture of the HepatAssist system, and on the left-hand side you see the schematic of the machine. During HepatAssist system therapy a patient's plasma, collected through an apheresis machine, is circulating through the bioreactor containing porcine hepatocytes. In the bioreactor the hepatocytes are segregated behind a hollow fiber membrane and perform many

of the metabolic functions of a healthy liver. This is the cross-section of an individual fiber, which is magnified, and the plasma is circulating through the lumen of the fiber. The cells are placed outside the membrane, and the membrane is not permeable to the cells. So, the case study on the PERV assessment will be focusing on this bioreactor.

[Slide]

With regard to the cells placed in the bioreactor, the cells are cryopreserved after isolation which allows for extensive quality control testing prior to use in the clinical setting.

[Slide]

This is the schematic of the quality control testing of cryopreserved hepatocytes, with the time scale which shows the time it takes to release cryopreserved cells for the clinical duration. In the blue boxes you see the critical steps of cell processing from herd qualification to cryopreservation, and the cells are released for clinical use only when all in-process and final release criteria are met. In-process and final release testing includes bacteriology, viral testing, microbiology and functionality testing.

[Slide]

It is a closed system and this is a picture of one aliquot of cryopreserved hepatocytes.

[Slide]

With regard to the treatment that patients are receiving with the HepatAssist system, there are 5 billion viable cells, hepatocytes that are introduced in each treatment and the hepatocytes in this system are segregated, as I said, behind the hollow fiber. Each bioreactor is 100 percent integrity tested, and the pores of the membrane are 0.15 micron size. Thus, the hepatocytes are not in direct contact with human cells during treatment.

[Slide]

The duration of the treatment is 6 hours administered daily to a patient, again, until either liver regeneration or organ transplantation. Based on current experience and the first study, on average there are 3-4 treatments per patient.

[Slide]

Now that I have introduced you to the system, I will just present a brief overview of in vitro studies studies -- PERV assessment; the strategies for risk analysis concern two parts, in vivo and in vitro testing. With regard to in vivo testing of patients, we have conducted three prospective testing on 29 patients' PBMCs. The testing was done by DNA PCR and all patients were found to be negative for PERV. The exposure time was 3 months to 5 years post-treatment.

With regard to the prospective testing, the testing is ongoing and it is a critical component of our protocol that all patients are to be tested at various time points. Patients' PBMCs are being tested by DNA PCR, as well patient serum is being tested by Western blot analysis.

[Slide]

With regard to in vitro testing, the hepatocytes were tested by cocultivation with 293 cells and there was no evidence of infectivity in human 293 cells cocultivated with human hepatocytes for longer than 35 days. Finally, we conducted a PERV assessment of the HepatAssist system.

[Slide]

The goal of this in vitro PERV assessment of the HepatAssist system was to test the system by the reactor that is used clinically for PERV infectivity and, second, to investigate whether the membrane in the bioreactor provides a barrier to PERV transmission.

As far as the study design, in order to simulate clinical conditions under which the system or the therapy is being delivered to the patients, we seeded the same number of porcine hepatocytes behind the hollow fiber membrane, and the hepatocyte bioreactor was perfused with culture medium. However, the duration of the experiment was 24 hours, which is 4 times longer than the clinical administration of the therapy. That was done to simulate the increased exposure to

the cells.

[Slide]

As a positive control, we placed PK-15 cells behind the hollow fiber membrane, and the design of that positive experiment was exactly the same. Five million PK-15 cells were used and the bioreactor was perfused with the same culture medium with 10 percent of serum, and the perfusion time was 24 hours.

[Slide]

This is a schematic of the experimental setup where the culture medium was used instead of plasma as in the clinical setting. It was perfused through an oxygenator and hollow fiber cartridge, and it was collected every two hours and then pooled together. The only difference with the clinical administration was that we did not use charcoal column as we believed that it could potentially interfere with the virus.

[Slide]

So, the medium was collected every two hours and then pooled together, and concentrated by a factor of 1000 by ultracentrifugation, where the cell supernatant from the cell compartment was not concentrated.

[Slide]

As far as the infectivity test, the circulating medium collected and cell supernatant were incubated with

human 293 cells for 35 days. As a control they used human 293 cells, and the positive control was human 293 cells infected with PERV.

[Slide]

In the infectivity analysis, at the end of the cocultivation period human 293 cells were analyzed for PERV infectivity by DNA PCR; with negative control, DNA from MRC-5 cells; and positive, genomic DNA from porcine PBMCs.

[Slide]

Now I would like to present the results of this study, first on the hepatocytes experiment. The media from the HepatAssist bioreactor containing porcine hepatocytes showed no evidence of PERV infectivity in human 293 cells inoculated with either cell supernatant or circulating medium which was 100 times concentrated.

[Slide]

As to the PK-15 cells, on the contrary, the media from the bioreactor containing these cells showed evidence, as expected, of PERV infectivity in human 293 cells inoculated with both cell supernatant or 1000-fold concentrate of the circulating medium.

[Slide]

However, in the PK-15 experiment the circulating medium showed at least a 5-log reduction -- and I want to emphasize it is a qualitative assessment in infectivity in

human 293 cells when compared to PK-15 cell supernatant in the bioreactor.

[Slide]

I would like to show you the picture of DAN PCR analysis for PERV infectivity, and want to point your attention to 4 particular lanes, lane 8, 8, 10 and 11. Lanes 10 and 11 represent PK-15 supernatant unconcentrated, incubated with 293 cells, and that represents a very strong signal. Whereby, lanes 8 and 9 represent concentrated 1000 times circulating medium incubated with 293 cells. Lane 8 -- there is no signal that could be seen on this autoradiograph, and lane 9 shows a very faint signal. So this is our qualitative assessment of reduction in infectivity.

[Slide]

In conclusion, the experiments with the HepatoAssist bioreactor containing hepatocytes showed no evidence of PERV infectivity in the human 293 cell line. Second, the HepatAssist bioreactor membrane reduced PERV infectivity by a factor of at least 5 logs using PK-15 cells.

[Slide]

Thirdly, the membrane based bioartificial organs may help, it is our belief, to reduce the risk of zenozoonosis such as PERV transmission. Further, membrane

based bioartificial organs segregate xenogeneic cells, thus preventing direct contact with patient tissues. Finally, cryopreservation of animal cells allows for conclusive quality control testing in this setting.

[Slide]

Now I would like to acknowledge my colleagues at Circe Biomedical and people we have collaborated with in the past, Primedica Corporation and Q-One Biotech. Thank you very much for your attention.

DR. AUCHINCLOSS: Thank you. We will move on now to a presentation fm the CDC by Walid Heneine, and I probably massacred the way you pronounce your last name.

Surveillance of PERV Infection in Exposed Persons

DR. HENEINE: Thank you for inviting me.

[Slide]

I would like to start first by going over some diagnostic strategies that we are using at the CDC to look for PERV infection in exposed persons or animal models, and then move quickly to the applications of these assays and what data we have obtained by using those assays.

One major point in the discussions today is the different strategies we could use, and what we have been doing, developing and evaluating at the CDC lab is both molecular and serologic approaches for the diagnosis. On this slide we see a listing of the assays we have to detect

PERV in the peripheral blood lymphocytes using primers and probes derived from preserved PERV sequences available to us to date.

We have also developed a PCR assay to detect pig-specific mitochondrial DNA to evaluate whether there is any evidence of microchimerism in the sample. What is not mentioned in the slide here is the assays we have using the PERV primers and probes to look for PERV RNA as a marker of productive infection or viremia, and we have applied those to the analysis of serum or plasma.

In addition, we recognize the importance of looking for antibodies against PERV as an adjunct approach for diagnosis, and we have also developed a Western blot assay which I will mention to you again later.

I have added here that in the case of a positive result by PCR and serology we should not stop there. We should also try to see if we can isolate virus from infected individuals.

[Slide]

The issue of microchimerism, as you have heard this morning, is very important for adequate diagnosis of PERV infection, and what we have developed is a simple method. The way we have adopted it is to use dilution of peripheral blood mononuclear cells and not DNA extracts of PBMCs. Rather, before extraction of DNA use dilution of

these peripheral blood mononuclear cells and then go back and test those dilutions for the presence of PERV sequences and for the presence of pig mitochondrial sequences, and then look at the titer of the positive signal that you will see.

Here, I have highlighted two scenarios that you may see. In the case of a chimerism the titer of PERV sequences will be equivalent to that of the pig mitochondrial DNA because it will reflect the presence of any pig cell in the dilution.

In the case of an infection you will see a higher titer of PERV sequence compared to the pig mitochondrial DNA sequences. This scenario, for example, reflects low level microchimerism in the presence of PERV-infected human cells. Again, I reiterate here that the key point is not dilution of the DNA but dilution of the cells, and we usually do a two-fold dilution so that you can very quickly determine what state you have.

[Slide]

Let me go back a little bit further to the serologic assay we have used and that we reported last month in Transplantation, with validation. The Western blot assay we use is a whole-cell lysate assay of 293 human cells infected with PERV. We use as a diagnostic marker the p30 activity of the gag protein. For seroreactive samples we

usually run another Western blot that has a control antigen, which is the uninfected 293 cells, to make sure that any reactivity we see is due to the viral protein and not to any non-specific reactivity associated with cellular 293 proteins.

This is an example of how we applied this assay on the evaluation of antibodies to PERV in pig islet-cell recipients which we have previously reported, as mentioned this morning.

[Slide]

I can go back and show you some of these assays that we have applied. For example, here, looking for PERV in humans that have received pig liver perfusions, we have looked at four patients. All four have been consistently negative by PCR. We have done the serology. The first three have also been seronegative. This is the time post-exposure. In patient "D", the fourth patient, we have seen a very weak seroreactivity at two months after the exposure but then afterwards we were not able to detect any reactivity at seven months, and we have now, two months later, another time point here. There was no molecular evidence of PERV in this patient, which led us to interpret this transient seroreactivity as not evidence of infection but maybe as evidence of exposure to viral protein.

[Slide]

This is the pig islet-cell recipients, the ten patients we have looked at. These are some characteristics that I would like to go over very quickly with you. This is the number of islet-cell clusters that they have received, and this is the evidence of prolonged survival seen in some of them, which was the detection of C-peptide.

We have also looked in a different way for evidence of xenograft survival by looking at the mitochondrial pig DNA in the plasma, and we have seen a strong correlation between detection of C-peptide and presence of pig mitochondrial DNA.

Nevertheless, despite the evidence of prolonged xenograft survival, none of the ten patients had evidence of PERV infection by the molecular and serologic tests that we have done.

[Slide]

So, so far from this limited surveillance, we can conclude that the data are reassuring. However, we would like to highlight that generalization of these results to other types of exposures is limited, and we need to evaluate PERV transmission by type of xenograft. However, our studies illustrate the minimum standard for laboratory surveillance for PERV.

[Slide]

I would like to move to another study we have done

that was mentioned by Carolyn this morning, which is looking at the evidence we found of PERV in porcine Factor VIII products. This study, actually, began when we looked for evidence of reverse transcriptase activity in the plasma of pigs. This was part of the Swedish collaboration we have had for pig islet recipients. When we looked at the plasma of the recipients for any evidence of RT activity that could be a marker of the presence of any retrovirus, we did not find any RT activity, however, when we looked at the controls, the pigs that we have tested, we detected very easily RT activity. These are duplicate results from two pigs. This is HIV-1 infected plasma. There were really equivalent signals.

[Slide]

So, that indicated to us that there is some level of viremia, as you have heard this morning, in pig plasma and, therefore, led us to analyze products of pigs that are made from pig plasma. We analyzed the porcine Factor VIII that is used now in licensed product for use for hemophiliacs.

[Slide]

Again, this is more specific information on the delectability of PERV RNA in pig plasma, and 18/20 that we have looked at were positive. Of these 18, 16 also had detectable RT activity by the Amp-RT assay. This is another PCR-based assay. Again, the correlation between detection of

both markers was positive, and that indicated to us that we need to look a little bit closer into this product.

[Slide]

So, we tested 13 lots of Hyate C -- this is the name of the product -- for both PERV RNA by RT-PCT and also we tested for RT activity.

[Slide]

We were able to detect the PERV RNA in all the lots tested, 13/13 for this sequence; again, 9/9 for the pol; and 5/5 for the env.

[Slide]

I will show you an example here. You see very strong signals here only when you reverse transcribe. When you don't reverse transcribe you don't see anything indicating that it is RNA in origin.

[Slide]

This is an example from the pol RT-PCR product.

[Slide]

We also wanted to see whether these sequences are associated with particles. As you have heard this morning, you can do that by looking at reverse transcriptase activity, which is a particle associated enzyme. We were able to detect RT activity in many lots. However, some of these lots had evidence of inhibition of the assay so we had to play around with the testing procedure by pelleting and

dilution of the product to be able to dilute out the inhibitor.

[Slide]

However, we were able to demonstrate the presence of RT activity in 10/13 lots tested.

[Slide]

As Carolyn said, we were also interested in seeing whether there is any infectious PERV and we carried out experiments by inoculating some of these lots into susceptible cells, 293, HT1080, and these cultures are still ongoing and, so far, we do not have any evidence of infectious PERV detected that could be isolated in these systems.

[Slide]

The key question here is do we see any evidence of transmission to hemophiliacs that have used this product? We collaborated with the Hematologic Diseases Branch at CDC that had previously collected a lot of samples from these recipients, and we tested a total of 111 samples and 88 were from the Factor VIII recipients and 23 were from unexposed hemophiliacs. We did blinded testing for antibodies to PERV.

[Slide]

None of the 88 samples were found to be positive; all were negative, as well as the 23 other controls. This is an example of one of these experiments about the

seronegative findings we have.

[Slide]

We could try to speculate on what could explain those results, and keep in mind here the scenario we have or the experiences we have in that population with the HIV-contaminated human Factor VIII, how HIV was transmitted to that population while, in this case, we have had no evidence of transmission so far.

[Slide]

So, you could look at the explanations for the lack of PERV transmission as related to the product itself, like possible presence of the infectious PERV -- possible absence of the infectious PERV in the product. We don't have evidence for that from the infectivity studies done by us and the FDA. This could be due to a little or no infectious PERV in the pig plasma originally. You have heard some data also from Gillian about the lack of any detectable infectious virus in pig plasma.

However, we still don't know very much about the negative findings in these assays, and what confidence we need to put into negative results using our knowledge today and the information in this culture system. So, we need to take this with caution and say that the negative finding may not confirm really absence of infectious virus.

We could also think that maybe there was some

infectious virus originally but that it has been lost or reduced during the manufacturing. The manufacture of that product includes cryoprecipitation, polyethylene glycol precipitation and freeze drying which all can reduce infectivity of retroviruses. However, this manufacture does not include any inactivation by heat or by detergent and this may explain how we were able to detect RT activity in these samples.

Also, there may be factors that are host related and that maybe PERV, which is present in this product, could have been infectious but then these PERVs were quickly inactivated by the complement system that we have that can be triggered by our naturally occurring anti-gal antibodies. So this could protect the hemophiliac recipients from infection.

[Slide]

I would like to stop here and recognize our collaborators at CDC. This is the team that is involved with all this work, and some collaborators also from other studies outside the CDC. Thank you very much.

DR. AUCHINCLOSS: Thank you very much. I am sure it is true for the other members, but my questions are building up for the end of the presentations but right now we are going to take a break for about ten minutes and we will start promptly at 10:45 to resume this session.

[Brief recess]

DR. AUCHINCLOSS: We will resume with a presentation from Diacrin by Jonathan Dinsmore.

Porcine Endogenous Retrovirus Testing in Patients with Pig Fetal Neural Cell Transplants

DR. DINSMORE: Thanks a lot.

[Slide]

My task today is just to present the patient testing data that we have generated to date. I wanted to spend a few minutes going over the application that we are involved in using pig cells, and we are using fetal pig cells and these are for treatments of contractable neurologic diseases. The cells are actually implanted stereotactically directly into the brain. Animal studies and patient data indicate that we may achieve and maintain long-term survival of these cells. These are stable grafts that maintained within the patients.

[Slide]

The patient data that I am going to summarize for you applies to three separate applications that are in clinical trials currently. One is for the treatment of Parkinson's disease. This is a Phase I safety study. It involves 12 patients. The transplant, as I said, involved fetal pig neuronal cells. These patients received transplants to one side of the brain. A total of 12 million

cells were implanted. There are two modes of immunosuppression utilized in this study. Six patients received systemic immunosuppression with cyclosporine and six patients received cells that were treated with a F(ab) fragment, a monoclonal antibody, and this is a proprietary methodology that Diacrin is developing.

[Slide]

The other trial that I will be presenting today is for a trial in Huntington's disease. This is a fatal disease for which there is no currently available treatment. Again, it is a Phase I safety study and 12 patients were involved. Actually, there is an error on this slide. The dose of cells was 24 million cells. Again, it was a unilateral transplant to one side of the brain. Again, two groups of patients, six receiving cyclosporin and six receiving antibody-treated cells.

[Slide]

A third trial that I will present patient data for involves the treatment of focal epilepsy. So, this is patients who have intractable focal epilepsy that are refractory to available drug treatments. Again, it is a Phase I safety study. There are three patients enrolled currently in this study of a planned six. Again, fetal pig neural cells with stereotactic delivery directly to the brain, four million cells. In this case, all the patients

received antibody-treated cells.

[Slide]

A little bit about the patient samples that were tested. These were tested at Primedica Labs in Washington. PBMCs were the test sample utilized. For the Parkinson's and Huntington's disease patients these trials were initiated prior to the institution of testing for PERV so we utilized our archival samples which were frozen at minus 70. For the epilepsy patients the trial was initiated after the initiation of PERV testing and, therefore, those patients were tested on a real-time basis as these samples were collected. For all of these, additional blood samples from the patients are reserved for additional testing, for serological testing or for testing with assays that may be developed as we go forward.

[Slide]

The assay procedure is simple. You have seen it before with the other presentations. DNA is extracted from the test sample. PCR is performed. We utilize the primers which recognize the protease region of the PERV. These were primers which were developed and published in 1997 in Nature Medicine. After the PCR, there is separation of the PCR products from the gel. It is then transferred to a membrane and then probed with a fluorescein tagged probe. After the PCR there is a third oligonucleotide which is used as a

probe on a Southern blot and the detection is via a light detection exposure to x-ray film.

[Slide]

These are the primers that we utilized. These are the PCR primers and this is the probe that is used on the Southern blot.

[Slide]

These assays were validated first before initiating testing on patient samples; to confirm that they were negative, these primers on human cells, human PBMCs and human cell lines with integrated human retroviruses. These same samples were concurrently run with positive controls for human DNA. They were all positive with human beta-globin primers. All of these samples were samples containing pig cells, either pig PBMCs from the pigs we used for isolating cells or from pig cell lines.

[Slide]

That is just summarized here on a table with the pig cells being positive for the PERV primers, the human lines all being negative but positive for the beta-globin sequences, and human samples that have various amounts of pig DNA spiked are all positive for both probes.

[Slide]

This is just an example of what you see on these Southern blots after running these assays. So, human PBMCs

are negative. Human PBMCs with various amounts of added pig DNA are positive. Pig PBMCs are positive, with various amounts of pig PBMC loaded. Human cell line and cells lines with integrated human retrovirus are all negative. Run on the same gel are the positive pig controls. So, with the validation of the assay we went forward with patient testing.

[Slide]

This summarizes the results for the Parkinson's disease patients as well as control samples which were taken from Diacrin employees. The time sampled is listed here. They are all negative for PERV sequences; all positive with the pig DNA spiked samples.

[Slide]

There were similar results for the Huntington's samples, again, taken at various times post-transplant.

[Slide]

Then, for the epilepsy patients, as I said, there is real time testing. So, baseline, three weeks, two months, three months and six months. More samples than this were actually collected but these are the samples that were tested, and they have all been negative. There is one missing here because a blood sample could not be drawn from the patient on that day.

[Slide]

This is just a representation of what one sees on the Southern blot analyses. There are duplicate samples here of patient PBMCs that are all negative; those same samples spiked with various amounts of pig DNA are all positive.

In addition to these patient tests that I have shown you, we have an ongoing Phase II clinical trial for Parkinson's disease. It is a blinded trial, therefore, I am not presenting the data on those patients here. PBMCs from those patients are being tested in real time and, to date, all of those samples have been negative as well.

One final note, although I am not going to present it here, we have done the cocultivation assay that you have heard about today with fetal pig cells, and those have been negative for the presence of any infectious virus being activated from the donor cells themselves. That is it.

DR. AUCHINCLOSS: Thank you very much. Our next presentation is by John Logan, from Nextran.

Development of Assays for Monitoring Baboons and Humans for the Transmission of Porcine Endogenous Retrovirus

DR. LOGAN: Thank you.

[Slide]

I would like to talk today about the development of assays for monitoring both baboons and humans for the possible transmission of porcine endogenous retrovirus sequences, and I would really like to split my talk into

actually three parts. In the first part I would like to talk a little bit about experiments done in vitro in cells and culture and coculture assays looking for potential transmission. I would also like to talk about looking in human patients who have been exposed to porcine livers and extracorporeal circuit. Then I would like to spend the last part of the talk talking a little bit about baboons that have been transplanted with various pig tissues.

[Slide]

To talk first of all about the issue of coculture, we performed a series of coculture experiments really to look at the possibility that transmission of PERV sequences from both hepatocytes and also stimulated lymphocytes from our transgenic pigs using human 293 cells as the indicator cell line. Our positive control in all these samples was PK-15 cells, either cocultured with 293 cells or virus produced from PK-15 pigs infected directly into 293 cells. The lymphocytes themselves were stimulated with PHA and IL2.

[Slide]

This is just a summary of that data. We actually analyzed the data in a number of different ways. We looked at both hepatocytes that were non-irradiated and irradiated hepatocytes, and stimulated lymphocytes, as well as PK-15 cells. We looked for the production of virus after 35 days of coculture in the 293 cells and we assays for the

production of infectious virus using standard RT assays, as well as RT-PCR assays of the supernatant at the endpoint of 35 days. All of these assays of hepatocytes, irradiated hepatocytes or lymphocytes from the transgenic pigs were all negative.

When we looked by DNA PCR at the end of the 35-day period, the results in the case of the lymphocytes were clear. There were no PERV sequences; no porcine genomic sequences. In the case of the non-irradiated hepatocytes in one set of experiments it was negative. In the other set of experiments it was positive for PERV sequences. The results were positive for pig genomic DNA. In the case of the irradiated hepatocytes, one set of experiments was negative. One was very weakly positive with PERV. It was variably positive for porcine genomic sequences.

Our conclusion essentially from this slide is that we certainly could see no evidence of the presence of infectious virus isolated from either hepatocytes or lymphocytes from pigs.

[Slide]

We then went on and we looked in various assays for both patient and baboon samples. We looked at two sets of assays. The first set of assays was the assays you have heard about in detail this morning, which are really PCR-based assays looking at various probes for the PERV genome,

either protease regions or envelope regions. We also developed an antibody-based test to try and look for the presence of antibodies, either in patients or various human populations or in baboons after transplantation of pig tissues, to see if that could be another methodology to look for potential evidence of exposure to virus.

[Slide]

If you look first at patients that underwent extracorporeal liver perfusion, in general the patients were all negative using PCR-based analysis of peripheral blood mononuclear cells.

[Slide]

There was one exception to that. That one exception we saw in two patients that were analyzed at the same processing time point but at different points, and those patients turned out positive in the PERV assay.

We then had a problem with getting enough DNA to see if they were positive for porcine genomic sequences and we couldn't equivocally say whether they were positive or negative. At subsequent time points for both of those patients were seen to be negative, and we felt the most reasonable explanation at that time was a contamination issue although, as Carolyn said this morning, we certainly could not exclude the possibility of a transient low-level infection.

[Slide]

We have modified our PCR technique in order to make sure we reproducibly get enough DNA for analysis. We actually store DNA now as well as lymphocytes. And, we have modified our DNA prep method for lymphocytes to get DNA more efficiently amplified.

[Slide]

Let me change gears a little bit and talk about the development of an antibody-based test. Clearly, the PCR-based technology is very useful in terms of level of sensitivity but can suffer from problems of contamination, and we wanted a second methodology that we could utilize to look at potential infection sites throughout the body, as well as a method which would give us complementary data in the case of a PERV positive. For that reason, we tried to establish some serological-based assays.

In the case of PERV this is a real challenge because, as you well know, there are no animal model systems which we can elucidate what potential levels of antibodies or time periods that we would obtain. So, we attempted to do this by trying to assemble various components of the assay in order to try and validate that we were capable of detecting anti-probe antibodies.

[Slide]

To do that we required an antigen source. In the

first instance we used recombinant gag protein in a number of different systems, bacterial and mammalian. We have produced anti-gag antibodies against the antigen produced in those systems. Then we tried to development an assay format using these antibodies as positive controls which would actually detect the presence of antibodies.

[Slide]

Let me turn now to the production of antigen. Just illustrated here are two systems that we utilized in E. coli. One of them was a fusion system with a gag coding sequence used to the protein A region. We could produce fusion protein containing gag. The second system was used as GST fusion, again with a recombinant gag sequence, and again, we could produce antigen and purified anti-antigen. Those antigens then were used for two different immunization strategies, one in mice for production of monoclonal antibodies and the other in rabbits for production of polyclonal antibodies. In addition to that, we also expressed the gag sequence in CHO cells as a separate indicator system for the development of antibodies against protein.

[Slide]

As we look at this system, what we have done here is we have taken two cell lines, one of Chinese hamster ovary cells, the second of Chinese hamster ovary cells which

actually express the gag antigen. In this case the gag antigen has a myc tag on it to verify that we could detect protein expressed in these cells.

In this case, this is mouse immunization. Obviously, the mice before exposure to the immunizing antigen has no detectable antibody either in CHO gag or CHO control cells. With immunization we see a specific increase in antibody binding to the CHO gag cells but not to the CHO control cells. In the case of the myc, that is just a control to show that we can detect this antigen produced in the cell.

[Slide]

We went on to look more closely at these antibodies. In this case we used five different test subjects. One was PBS media. one was a monoclonal antibody isolated against gag. One was rabbit pre-immune serum; and one was rabbit post-immune serum. With an ELISA type format, we then used five different antigens. We used Chinese hamster ovary cells; CHO cells with the gag protein; basic E. coli cells and E. coli cells that contained the GST gag fusion, as well as purified GST gag fusion protein.

We looked first at monoclonal antibody lanes. We looked first in CHO cells. Obviously, monoclonal antibody doesn't bind CHO cells. The CHO cells expressed in gag cells by significant binding. It doesn't bind to E. coli extracts

but it does bind to E. coli extracts containing the gag fusion protein as well as the purified gag antigen.

In the case of the rabbit pre-immune serum, we see no binding to any of the substrates. In rabbit post-immune serum, no binding to CHO cells; significant binding to CHO gag cells. However, in the case of rabbit post-immune serum, this is immunized with protein A gag fusion which has E. coli protein so we do see production of E. coli antibodies but there is some stimulation on the GST gag E. coli cell.

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This is just a summary of series of monoclonal antibodies that we have isolated against the gag protein, the polyclonal antibodies that we have utilized. All of them bind in an ELISA type format. Some of them bind quite well on the Western blot format, and some of them actually identify infected 293 cells in fluorescent-based cell assays. So we felt we now had a good resource of antibodies in order to start the process of trying to develop an antibody-based test.

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We expressed three different PERV gag fusion proteins. We confirmed integrity of the gag reading frame by sequencing peptides. We then immunized mice and rabbits with this fusion protein.

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We tried to development an assay. We decided initially to utilize PERV-infected human 293 cells as a source. Clearly, the PERV-infected 293 cells produced more of the gag antigen, produced envelope and, therefore, in theory we could see production of many different potential antibodies produced.

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This is just a very quick look at some data to show that we can, in fact, detect specific binding to PERV-infected human 293 cells, and these are 293 cells obtained from Robin Weiss' group. We looked at rabbit pre-immune sera. There is no binding to either uninfected 293 cells or to 293 cells which have been infected with virus and PK-15 cells. With rabbit post-immune sera, no binding to 293 cells, but significant binding to infected 293 cells. And, the same with monoclonal antibodies, no binding to 293, good binding to 293 infected cells. So, we thought that these cells would be good in an ELISA type format initially just to look for the production of antibodies against PERV.

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We decided to do that initially looking at patient samples, and this is actually looking at patients' post-liver perfusion. We are actually looking at post-allografts. These patients are on immunosuppression. We looked initially at the anti-porcine response that these patients have

against pig cells in general and not just against PERV. What we see is that in the first few day post-perfusion a rapid induction of anti-gal antibodies with IgG and IgM. We actually failed to detect any significant level of binding to other pig antigens. So, the antibody response seemed dominated, at least in this case, by alpha-gal antibodies. So, these patients saw porcine tissue, and that porcine tissue stimulated an immune response.

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We looked now in an ELISA type format to see if these patients over time had expressed antibodies which could now recognize PERV-infected cells. We looked first with PK-15 virus-infected 293 cells. This is rabbit serum pre-immune and post-immune, and we see a significant binding to 293 cells. If we now look at these patient samples over time, both IgG and IgM, we see no difference in binding between uninfected and infected 293 cells.

If we look in a Western blot type format where we now take extracts from 293 cells, either infected or uninfected, and we also do an additional test where we take uninfected 293 cells and add pertussis toxin -- we add pertussis toxin to show that these patients can, in fact, have antibodies that we can detect.

We looked first in the case of the monoclonal antibody to look at infected 293 cells versus uninfected 293

cells. We see binding to gag-related peptides in infected cells but not uninfected cells.

What is consistent in this patient and also in the other patient is that we no specific binding or no antibodies with specifically recognized bands present in only 293 infected cells as opposed to 293 uninfected cells. We do see good binding to pertussis toxin.

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We also went on and apart from analyzing these patients, we also looked at the human population in general in a small part, and we looked at two given populations. We took a random human serum population, and also a population which should have greater exposure to pig cells. In this case, we looked at swine practitioners. These are veterinarians who specifically work with swine on a day-to-day basis and have reported during the last year to have lots of blood contact with pigs.

If you look in an ELISA type format again, these are the control antibodies. We see good binding only to infected, not uninfected 293 cells. But in case of either the random human population or the swine practitioner serum we see really no evidence of any difference in binding between infected and uninfected 293 cells.

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We have also done that same experiment using a

Western blot format. I show you a few examples of that here from the random population as well as the swine practitioner population and, again, there is no difference in binding between infected or uninfected 293 cells.

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We then also went on to look at pig to baboon xenografts -- and I told you the xenografts have actually been done some time ago -- to look at DNA PCR-based techniques as well as antibody-based techniques to see if we could detect presence of PERV sequences. Realizing that there is some controversy over whether PERV could infect baboon cells or not, and I think we have heard today results both ways in this particular assay, at least with regard to one primate species, we felt, however, it was still worthwhile to look at these samples rather than to wait to have conclusive evidence either way.

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We looked first of all, and this is just one example of many that we have done -- we decided to adopt a slightly different regime in which we looked at xenografts that in this case had only been in for a few days, and then looked at both the antibody response of the baboon against the xenograft after a heterotopic heart transplant or the response against specifically PERV antigens in 293-infected cells. After removal of the xenograft, the baboon in this

case was maintained on immunosuppression for a further 90 days. So, we put the xenograft in with immunosuppression, removed it and continued the immunosuppression.

What we see is that there is actually a lack of fairly strong anti-pig response in these baboons against pig tissue. It is dominated again by alpha-gal response. I only show the alpha-gal response in this case.

However, if we look at the ELISA technique to pick up potential anti-PERV antibodies, the controls show good binding again. However, in the case of various time points, looking at 293 infected and uninfected cells, we see no difference in binding. So we see in the baboons again an anti-pig response showing they can respond to pig antigen but no anti-PERV response.

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We have also looked by DNA PCR. In this case we show lymphocytes. This is just an indication of the sensitivity. It essentially goes down 1 cell in 10_6 cells. We can detect the PERV sequences. In this case, we show 3 baboons here, and these are baboons before transplant and 90 days after transplant, and we see no evidence of PERV sequences on the lymphocytes. We are following this up and looking at multiple tissues throughout the baboons but that analysis is not yet complete.

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We have looked, in this particular case, at longer surviving grafts. Again, 90 days post-transplant with constant immunosuppression we see no evidence by PCR probe sequences on lymphocytes.

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The conclusion then in terms of an anti-PERV summary is that we really see no evidence of anti-PERV antibody either in the normal human population or in swine practitioners. In the baboons we have looked at today, again, no evidence of anti-PERV response in antibody-based tests; and the same thing in terms of control and patient serum.

Clearly, we will continue to expand these studies to look at multiple tissues in baboons at multiple different time points with longer surviving xenografts, and we will continue to follow-up the data. Thank you very much.

DR. AUCHINCLOSS: Thank you very much. To conclude the morning's presentations, we will hear from Khazal Paradis, from Imutran, on retrospective patient studies.

Retrospective Patient Study - Testing Strategy and Methods

DR. PARADIS: Good morning. I first want to apologize that I am going to be presenting again just the patients that are participating in this study, which we have code names XEN 111, but I am not going to be presenting the results. I particularly want to apologize to those who have

heard this apology several times.

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XEN 111 is a study that had multiple testing labs, many collaborators, and has taken a lot of time to get everything together. The objectives of the study were to detect circulating endogenous porcine retrovirus or antibodies to PERV in subjects who had been in intimate contact to living porcine tissue. So the first aim was to search for evidence of transmission of PERV in those who had been in intimate contact.

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The secondary aims were to search for horizontal transmission or human to human transmission of PERV in intimate contacts of patients who had been found to be positive for PERV by either antibody or PCR screen.

Then we also looked retrospectively to see if there were any unusual signs or symptoms in patients who had been found to be positive by PCR or antibody screen.

Finally, in those who had been found to be infected we would then look to see at the source pig line, the sequence of the PERV and compare it to the PERV that we would find in the patient.

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The methods -- after a informed consent we obtained a short medical history and a questionnaire on

exposure to pig tissue. Peripheral blood mononuclear cells and serum were prepared and aliquoted at the sites, frozen at minus 70 and then shipped on dry ice to the testing labs.

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In terms of patient samples, we have 100 patients that come from St. Petersburg, Russia where they have a particular extracorporeal splenic perfusion for what they call immunotherapy. This is usually severe burns or cancer. These patients had their perfusion either on the same day as when we took the sample or up to almost ten years before.

We had 28 patients who were treated with the HepatAssist device that you heard Zorina Pitkin talk about earlier today. These patients come from the United States, France and Israel. We had 15 patients who had skin grafts up to 10 years ago, from Germany. Then there were 14 patients who had received pig islet cell transplants, and 9 of them were in association with a kidney transplant, and these patients have been immunosuppressed since. These come from Sweden and Auckland, New Zealand. We then have 2 patients who participated in an experiment with extracorporeal kidney perfusion. These come from Sweden. These lasted for 15 minutes and 65 minutes. Finally, we have 1 patient from Montreal, Canada who received extracorporeal perfusion through a whole pig liver for about 4.5 hours before having a successful liver transplant.