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CENTER FOR BIOLOGICS EVALUATION AND RESEARCH
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Topic II: MLV-Related Human Retroviruses and Blood Safety

We have a very busy afternoon on, as said before, XMLRV and MLV-related retroviruses as they affect blood safety. We have four questions for the committee, only one of which will require a vote, which is the first one. The others are for comments and discussion only.

Let's begin the second topic of the day. Dr. Indira Hewlett is going to give us an introduction and background.

By the way, I will also mention to you that we are switching two of the sections. B and C will be switched, so we'll go to the recent studies next and then follow it with a summary of current research.

Agenda Item:
Introduction and Background

DR. HEWLETT:

Thank you, Dr. Hollinger, and good afternoon, everyone.
In this afternoon's session, we will be focusing on murine leukemia viruses that have been identified in humans and their potential impact on blood safety. The issues that FDA is specifically seeking the advice of the BPAC on are donor deferral based on a history or diagnosis of chronic fatigue syndrome, CFS, or prostate cancer, with which these viruses have been associated; and testing for the newly identified MLV viruses in humans, recognizing that disease causation has not yet been established.

The MLV-related retroviruses were first identified in humans in the DNA of prostate cancer tissue using a microarray of conserved viral sequences. The sequences found in prostate cancer were clustered with the xenotropic murine leukemia viruses, and therefore were termed "xenotropic murine leukemia virus-related virus," or XMRV. Subsequently, polytropic MLVs were also identified in humans. Together, these viruses will be referred to as "MLV-related viruses," for the purposes of today's discussion.

In 2009, a U.S. study reported detection of XMRV in the blood of 67 percent of CFS patients and 3.7 percent of healthy controls using PCR. These viruses have since been detected in the blood of up to 14 percent of healthy controls. However, several studies have shown a lack of detection in CFS, prostate cancer, and blood donors. Finally, although disease association has been reported, causation has not yet been established.

In July of this year, we held an informational session of the BPAC where a number of topics were discussed, including the identification and detection, or lack of detection, in CFS, prostate cancer, and blood donors. Assay validation study plans and assay development efforts were presented.

We learned about the AABB recommendation for use of donor education materials on CFS and indefinite deferral for donors who voluntarily disclose their CFS diagnosis. We also heard about Health Canada's policy of indefinite deferral for prostate cancer and recent introduction of deferral for those who voluntarily disclose their CFS diagnosis.

Since then, there have been a number of reports that have presented either positive or negative findings in regard to disease. In the case of CFS, at least three reports are worthy of mention. I will not discuss them because you will be hearing about them later in this session -- in fact, right after my talk. The most important point that should be mentioned here is that, at least in one of the studies, up to 86.5 percent positivity was observed in CFS patients. This is using PCR on PBMC DNA. The virus identified was polytropic MLV. About 6.8 percent positivity was observed in U.S. healthy volunteer blood donors.

However, in contrast, a number of negative findings have also been reported. They are summarized on this slide. There are at least two studies in the U.S. and several from Europe and one from China where, by using various methods, such as PCR, Western blot, ELISA, serology, and virus culture, no XMRV was detected in CFS cases, healthy controls, and blood donors.
In regard to prostate cancer, there are at least three reports that show positive association. Petros et al. reported at the First International Workshop on XMRV the detection of XMRV sequences by PCR in three prostate cancer tissues from three separate patients. In Northern Europe, when 355 prostate cancer tissues were tested, two out of 355 were positive for XMRV by PCR. All of the PBMCs from the 40 patients for whom PBMCs were available were negative by PCR, as also were all healthy controls.

Finally, a study that was recently published by Danielson et al. reported 22 percent positivity of XMRV sequences in 144 prostate cancer patients from the southern U.S. In this study they used PCR to look for XMRV. In the same study, both normal and tumor tissues were available for a subset of cases, and XMRV sequences could be detected in both normal and tumor tissues. Again, in contrast to the positive findings, there are a number of negative findings that have been reported, one study in the U.S. and three studies in Europe, where they looked at a number of different prostate cancer tissues using RT-PCR, immunohistochemistry, serology, and virus culture.

In regard to transfusion, transfusion transmission of these viruses is theoretically possible due to the presence in blood cells and the evidence of cell-free virus. Also low but detectable transient viremia has been reported in rhesus macaques at 4 to 14 days and seroconversion between 11 and 14 days post-intravenous inoculation.

A seroprevalence of 1.7 percent has been reported in Japanese blood donors, .1 percent in U.S. blood donors, and by PCR, up to 6.8 percent positivity has been reported.

Again, in contrast, however, no evidence of XMRV was found in two other U.S. studies of blood donors, one of 121 blood donors where PCR and serology were used and a different U.S. of up to 1,500 blood donors that were tested using a TMA assay. No XMRV sequences were detected in 105 plasma, 19 PBMCs in Cameroonian blood donors and HIV-infected individuals in Uganda. As you can see, there are a number of conflicting findings in regard to CSF, prostate cancer, and blood donors. The potential sources of these discrepancies could arise from a number of factors, such as the differences in study populations, geographic differences in prevalence, differences in case definition criteria and stages of illness, specimen processing, sensitivity and specificity of the test methods used in the studies, potential genetic variation of the virus and other unknown factors. Therefore, a need for assay standardization and validation was identified.

During the past year, there has been a great deal of effort in this direction. This effort has been spearheaded by the National Heart, Lung, and Blood Institute, under the leadership of Simone Glynn and Jerry Holmberg, who have created a scientific research working group composed of scientists from the government, academia, and blood organizations to validate assays for transfusion-transmission studies. The Blood Systems Research Institute is coordinating the laboratory efforts of this group by putting together analytical and clinical reference panels, to be evaluated by multiple labs, including the CDC, NIH, the Whittemore Peterson Institute, Blood Systems, and two labs at the FDA, for testing of blood donors in future studies. In addition, FDA is currently developing NAT and serology panels for future lot release, if needed. Some of you have participated in these studies.

Finally, the NIH, through the NIAID, is sponsoring a proposed study on XMRV and related viruses in chronic fatigue syndrome. This study will include a number of different laboratory sites and clinical collection sites, and will use various sample-preparation methods and assays to look for XMRV in CFS. The study is being coordinated by Ian Lipkin at Columbia University, and it has a number of experts that are inputting into the design of the study.

In regard to blood donation, no direct evidence for transfusion transmission or association with a transfusion-transmitted disease exists. Therefore, FDA has not established donor policies specific to the MLV viruses. FDA regulations require donors to be in good health at the time of donation, and medical directors exercise judgment on whether CFS patients meet those criteria.

In regard to prostate cancer, a large survey in Sweden and Denmark showed no increase in disease cases in more than 800,000 blood-transfusion recipients without a prior cancer diagnosis at the index transfusion. Indefinite deferral of donors who voluntarily disclose their CFS diagnosis is in place now in Australia and New Zealand, the U.K. and parts of Canada. In the U.S., the AABB is recommending the use of donor education materials on CFS and indefinite deferral for donors who voluntarily disclose their CFS diagnosis.

With that brief background, I would like to put forward the questions that we are posing to the committee:

- First, do the scientific data support asking donors about a medical history and/or a diagnosis of CFS as a basis for indefinite deferral?
- Second, we would like the committee to discuss whether the scientific data support asking donors about a medical history and/or a diagnosis of prostate cancer as a basis for indefinite deferral.
- Third, we would like your comment on the scientific evidence that would be needed to justify a policy of donor testing for infection with MLV-related human retroviruses. In particular, should donor testing be considered in the absence of confirmed disease causation?
- Four, assuming that testing is warranted, please comment on the potential utility of NAT and/or serologic testing of blood donations to ensure safety of the blood supply from transmission of MLV-related retroviruses.

To assist in the discussion, we have put together a session which has a number of presentations on MLV/XMRV. They include:

- A review of current data on the virus, including highlights of the First International Workshop.
- Research updates and current data on the association of these viruses with CFS.
- Data from animal infectivity studies.
- An update from the Blood Scientific Research Working Group on assay validation using the MLV and XMRV panels.
- A proposed design for prospective and retrospective donor prevalence studies that is in the planning process.
- An update on assay-development efforts on MLV-related viruses.

I'll close with that. Thank you for your attention. I will take any questions at this time.

DR. HOLLINGER:

Thank you.

I think we will to the next -- because we'll be discussing much of what you presented here, Indira.

The next section we are going to discuss is "Recent Studies of Epidemiology of MLV-Related Human Retroviruses." The first talk will be by Dr. Lo, in which he will discuss a U.S. study. I want you to be sure to pay attention to the timer.

Agenda Item:
Recent Studies of Epidemiology of MLV-related Human Retroviruses

DR. LO:

Ladies and gentleman, my name is Shyh-Ching Lo.

While we are waiting for the slides, I would just like to say, because the results of our study have already been published in a PNAS article more than two months, I'm quite sure many of you have had a chance to read it. So I'm going to just very quickly summarize our study results.

I would very much like to start by saying, why did we get into these XMRV studies? Because I transferred to FDA only about two years ago. Before I came to the FDA, I served as a staff pathologist at AFIP, the Armed Forces Institute of Pathology, for more than 23 years. During that time, in the early 1990s, a research study in my laboratory at AFIP led to the discovery and the characterization of previously unknown human mycoplasmas in patients with AIDS. Subsequently, several laboratories' studies reported that these mycoplasmas, which we called AIDS-associated mycoplasmas, were associated with the development of chronic fatigue syndrome. That was in the mid-1990s.

So blood samples from CFS patients followed by specialized centers or individual clinicians -- they sent their samples to us for the mycoplasma studies.

However, our subsequent study concluded that we did not really see any evidence of an association between this mycoplasma that we call Mycoplasma fermentans or Mycoplasma penetrans and the development of CFS.

Portions of the CFS blood samples sent to us had been maintained in frozen storage at -80. We thought this provided a very unique opportunity for us to study for the evidence, if any, of XMRV infections in CFS patients. This is obviously in the early part of this year, after the many conflicts in results had been reported of XMRV in the patients with CFS.

Overall, most of our CFS patients were sent to us by Dr. Komaroff, at Brigham & Women's Hospital, Harvard Medical School. He provided us 29 blood samples obtained from 25 CFS patients. In addition, we have 12 blood samples also sent to us from different centers and clinicians. So in this particular reported study, we studied 41 blood samples obtained from 37 patients with CFS. All these specimens had been frozen at -80 since the mid-1990s.

Our collaborator, Dr. Harvey Alter, at the Clinical Center, NIH, provided previously frozen PBMCs from 44 volunteer healthy blood donors. These samples were collected between 2003 and 2006.

The results, as we reported in PNAS: We found that a very high percentage of the patients tested positive by a nested PCR targeting the murine leukemia virus gag gene. In comparison, about three out of 44 -- so 6 to 7 percent -- of the volunteer healthy blood donors also tested positive.

This is the gel electrophoresis of the PCR amplification products. As you can see, using this primer design originally reported in a Science article -- using the primer sets, amplify 731 bp products. Most of our products come out slightly larger than the expected size of the 730 bp. These all turned out to be 736 base pairs, because we did not do this as specific deletions identifying the XMRVs.

Coupling with the second round of PCR, with 45 cycles, using the internal primer set -- this is the in-house primer set we have, NP116 and NP117 -- the product of 380 bp was amplified -- more than 80 percent of the patients, anyway.

So in the first round of PCR, we identified about 40 percent from the first 25 CFS patients, PBMC. This was provided by Dr. Komaroff.

I would also like to mention that virus RNA could also be detected by RT-PCR. However, the sensitivity apparently is lower, and we required a second round of PCR to detect them, and also fewer patients tested positive by RT-PCR. But this could also be because our current protocol of RT-PCR assay is less sensitive than our PCR assay for the PBMC DNA.

This is for comparison for 25 healthy blood donors, PBMC DNA. In the first round of 40-cycle PCR amplification, we only see one of the patients that has positive amplicons.

Coupling with a second round of PCR, the 45 cycles using internal primer PCR amplification -- these are the same patients -- only this patient could be confirmed. Also this particular patient's product could be confirmed.
As you can see, there is a mini side bands that have been amplified, and all this band was a close size to the target 413 PB. In this case, they have all been retrieved for sequencing. All the sequences turn out to be human sequences. Only this one and this one is murine leukemia virus gag gene sequence.

This is the alignment of the amplified gag gene sequences. This is from the CFS patients from type 1, type 2, type 3. Most of those sequences belong to the type 1. But as you can see, all these sequences missing this specific 15 nucleotide deletion are reported in the XMRV sequence, and the sequence alignment certainly is much more closely aligned with polytropic murine leukemia virus and has more variation with the XMRV sequence. But this is only the first 184 base-pair alignments.

Using the phylogenetic analysis, as you can see, all of the sequences we obtain from the CFS patients cannot be grouped in the same cluster as the XMRV. The cluster is actually more closely related to the polytropic virus sequence, including the blood donors' sequences.

When we translated those sequences into amino acid sequences, this is again to show all these amino acid sequences cluster better with the polytropic virus, but also closer to the XMRV.

If we compare to the ecotropic virus, the murine leukemia virus, this is the virus often being used in the laboratory to study murine leukemia viruses, like Friend leukemia virus and Moloney murine leukemia virus -- very distant from the sequences we obtained.

In some of the patients -- a very few of them -- we successfully amplify the envelope gene. The envelope gene sequence, again, clusters better with the polytropic murine leukemia virus, and much more distant from the XMRV sequence. Once we had these results, we had to decide what we were going to do with the findings. Certainly we debated for a while. This is basically based on a PCR amplification assay, and in all PCR-based studies we should have a concern of contamination. In this particular case of the XMRV study and MLV-related viruses study, we have three main concerns of contamination.

The first one is, can this be a contamination by the PCR amplicons because of so many sequences being amplified in each amplification? Can that be a contamination source? Since we see more than six different sequences in this study, the variations certainly don't(?) favor that this can be coming from the PCR amplification contamination. During this study, we built in 300 to 400 negative controls, running in parallel. We have never amplified any MLV sequences in our negative controls.

Can this be a contamination by murine leukemia viruses commonly used in the laboratory? We also thought this was not quite likely because the sequences are quite distant from all those ecotropic viruses commonly studied in the laboratory. It's very different from the viral vectors normally studied in the laboratory.

The third question is the more difficult one: Can this be a contamination by mouse DNA? The mouse DNA genome contains endogenously many copies of the related retrovirus sequences. We thought this would be necessary for us to develop an assay to verify that there is no contamination of mouse DNA in the assay system we use and also in the clinical samples that test positive for MLV-like virus gene sequences. We developed a highly sensitive nucleic acid PCR assay targeting mouse-specific DNA. In this case, it's a mitochondria DNA, because we need a target gene sequence that is very well conserved among all the different species of mouse and also has multiple copies in each of the mouse cells. That's why we selected mitochondria DNA, because each cell carries 200 to close to 2,000 copies of these genes.

I'm not going to go into the way we designed it, but I just want to show you that the semi-nested PCR assay targeting the mouse-specific mitochondria DNA allows us to detect 10 femtograms of mouse DNA in the first round of the PCR, without a nesting step. This can detect 10 femtograms in the presence of the background 35 nanograms of human DNA. In parallel reaction, as you can see here, our gag gene PCR assay would be able to amplify - very faint band in the 10th picogram of mouse DNA.

So it's almost 1,000 times more sensitive than our gag gene PCR amplification to amplify the mouse DNA.

Coupling with internal nested PCR, this mitochondria-specific PCR, allows us to detect 2 to 3 femtograms of mouse DNA. In the background again is a spike into the 35 nanograms of human DNA. In this case, the internal PCR amplification of the gag gene allows us to detect about 500 femtograms of the mouse DNA.

Using this assay, which is close to 1,000 times more sensitive than the gag gene PCR assay, we come back to verify that all the DNA from those CFS patients, particularly those where we can detect murine leukemia virus-related gene sequences in the first round of PCR -- that's supposed to have higher titers of this viral gene sequence -- none of them show evidence of the mouse DNA contamination. In a parallel run, we showed the assay did detect 10 femtograms of mouse DNA in the background of 35 nanograms of human DNA in the first round of PCR. Coupling with the second round of PCR again, we can detect 2 to 3 femtograms of mouse DNA in the background of 35 nanograms of human DNA. Again, we did not detect any mouse DNA in all of the CFS patients and the healthy blood donors that tested positive for MLV-like gag gene sequence.

We also went back to check -- all of those patients' blood DNA was preserved, stored since the mid-1990s. Eight of these CFS patients' previous frozen blood samples tested positive for MLV-like gene sequence. We went back to get their blood. This was by Dr. Komaroff. Seven of these eight repeat blood samples -- this has been processed freshly and tested freshly -- also tested positive for the PCR assay for this murine leukemia-like gag gene sequence. However, the viral gene copy number in the repeat blood samples did not really increase. On the contrary, they appeared to be lower than those found in the previous blood samples of these patients. The gag gene sequence showed a quite noticeable variation in the blood obtained from most of these patients 15 years later. The details of that comparative analysis will be reported separately.

I'm not going to go into this. After our publication, there were several publications, some that could not find XMRV in chronic fatigue syndrome patients, could not find in HIV patients by some studies, as described, but found in prostate cancer patients. This depends on specific PCR conditions. I would like to conclude our study by saying that our results support the earlier finding that MLV-related virus gene sequence could be found in the blood of many patients with CFS. These viral sequences could also be detected in a small fraction of volunteer healthy blood donors. But differing from the reported finding of near genetic identity of all XMRVs in patients with CFS, in prostate cancer, and also in healthy blood donors, our analysis of the viral gene sequence revealed a more genetically diverse group of MLV-like viruses. The viral gene sequences were more closely related to the polytropic murine leukemia virus.

So our study shows that the zoonotic MLV-related viruses are infecting some human beings and that the disease association and possibility of blood transfusion by this murine leukemia virus-like retroviruses in human will require further studies. I would like to acknowledge those scientists, Dr. Li, Dr. Pripuzova, and Dr. Hung, in our laboratory doing all the work. Our collaborators, Dr. Harvey Alter and Dr. Wang, at the Clinical Center provided us those healthy blood donors' samples. Dr. Komaroff, from Harvard Medical School, provided most of the CFS patients. Dr. Puri and Dr. Witten are my superiors in my office, OCTGT. I would also like to acknowledge Dr. Epstein for his strong support of our work.

Thank you very much.

DR. HOLLINGER:

Thank you, Dr. Lo.

Questions for Dr. Lo? He is going to have to leave. Yes, Ken?

DR. NELSON:

Two questions.

I'm interested in the blood donors who tested positive who were healthy.

Was there any epidemiologic data from them that they had any association with people who had chronic fatigue syndrome, household or otherwise?

Secondly, apparently you didn't look for an immune response to the XMLV -- or maybe you did; I don't know. But I think another paper reported this. I wonder if you would comment on that.

DR. LO:

Yes. For the first question -- did those healthy blood donors have any association with or were related to any CFS disease patients? -- we could not really answer that. That's just random healthy blood donor samples provided by Dr. Alter. Dr. Alter, in later discussion, I'm quite sure, can answer more about this. On the antibody response, I think there later on will be speakers who will talk about it, particularly in the healthy blood donors.

DR. COFFIN:

We have had some of this discussion before, but I would like to raise some issues again, which will be familiar to you, for the sake of the rest of the committee.

First, I have to point out, of course, that unless you had something new to tell us, as yet you have no virus that goes with these sequences. Is that correct?

DR. LO:

There's no virus isolation --

DR. COFFIN:

You have only PCR-isolated sequences?

DR. LO: Right.

DR. COFFIN:

Second, although I think I may have originally suggested mitochondrial DNA to you as a way to test for contamination, I worry very much about it, for a couple of reasons. One is that, of course, if there is contamination of DNA from some source -- you point out that there is a wide variation in the number of mitochondria per cell -- you have no idea what the cells are giving rise to that contamination, much less any idea of how many mitochondria you might have. PBMCs, for example, have relatively small amounts; muscle cells or oocytes have huge numbers of mitochondria. You have no idea. Second is that your extraction procedures may, in fact, differentially extract mitochondrial DNA from genomic DNA.

For these reasons, my lab has developed an assay, based on another repeated element, that presents about 1,000 copies per cell that I think gives much better results and can detect mouse DNA contamination in many samples where mitochondrial DNA doesn't. In fact, using that assay, I have two collaborations ongoing which have led to papers that are going to be published by the end of this month in Retrovirology that show, in a sense, results similar to yours. In one case, two sets of samples, one from CFS patients and one from non-contemporaneous controls in fact, like you have -- showed that one set was highly positive for almost exactly the same sequences in fact, I think in some cases exactly the same sequences -- that you report and in the other case was completely negative. In that particular study, those were again taken at different times. In fact, it was the CFS patients that were largely negative and the control samples that were taken later that were largely positive. In all cases in that study and in another one in which I'm a collaborator, the samples that proved to be positive were positive for the IAP sequence control that we use for mouse DNA contamination. We attribute those results -- I think the only reasonable thing to do is to attribute those results to contamination with some laboratory reagent or another, with very minute traces of mouse DNA. One-one hundredth of a cell is enough to contain an amplifiable provirus, although your assay seems to be quite a bit less sensitive than that.

Not all of the samples were positive for mitochondrial DNA, in agreement with the idea that this is actually a less sensitive assay than IAP for mouse DNA contamination. So I worry very much about that. There are a couple of things that you could do that would help reassure me. One is -- and again, I think we have had this discussion -- the sequences that you obtain are from bulk PCR product. A hallmark of the sequences that you would be amplifying from contaminating mouse DNA -- there would be a wide variety of them, each one of which might correspond to a previously sequenced and identified provirus, within each band. You would not see that in a bulk sequence. You would just a computer-generated sort of average of what was in there, a recombinant of some kind of what was in there. Have you actually gone back and done that, cloned or done limiting dilution amplification on those to show that those are monomorphic?

DR. LO:

On mitochondria DNA and IAP sequence PCR. We did compare the sensitivity of our assay. Obviously, in our hands, and I think in some of the earlier presentations from your associated group -- I think the mitochondria PCR assay is much more sensitive in detecting the mouse DNA if we run in parallel. That's why we continue to use the mitochondria DNA. In this case we did not use the high-mitochondria-containing mouse cell, like the muscle cells. We are using the spleen cells. That's the most common that people use for the laboratory study. It's hard for me to believe that we had a selective isolation of the mitochondria DNA and did not have that nuclear DNA being isolated. In fact, those kits many publications said isolation is equally efficient with the nuclear DNA and mitochondria DNA, in previously published studies.

Of course, the contamination always is a concern, like you said, because the sequence is so closely related to some of the genomic sequencing. But I would also like to say that we amplify and sequence many of those mouse DNA amplifications and we very rarely see the intact product from the PCR sequence. They always have many different interruptions of the stop codon. Of course, you can say maybe it's amplified -- for so many different sequences, they are specifically amplifying particular sequences. We cannot completely rule that out. But essentially all the sequences that were obtained from the patients have the intact reading frame.

DR. COFFIN:

But there are some internal inconsistency, I think, in your results. You claimed a 1,000-fold greater sensitivity of mitochondrial DNA compared to MLV-based amplification, but yet there 100 MLV proviruses. At a 1,000-fold greater amount, that would require 100,000 mitochondrial DNA copies. I don't think anybody believes there are that many mitochondrial DNA copies per cell.

DR. LO:

That's why we use the spleen cells. The spleen cells obviously have a very low concentration, a very low number of mitochondrial DNA. We did not select those, like muscle cells, that have very high copy -

DR. COFFIN:

The data you showed were from the spleen cells? You were claiming 1,000-fold difference.

DR. LO:

No, no, no. We are saying the reason we select the mitochondria is because mitochondria have multiple copies in each cell. That's why we use those targets. The reason we select spleen cells, of course, is because the mitochondria concentration is much lower. We use that as our assay.

DR. COFFIN:

But you can still see it in 2 to 3 femtograms of spleen cell DNA, where it takes 500 femtograms to see -

DR. LO:

Right, putting it into 35 nanograms of human DNA, we can detect 3 femtograms of those mouse -- most of the DNA, of course, is coming from the nuclear DNA. It's not from the mitochondria DNA.

DR. COFFIN:

You didn't reply to the second half of my question, which is, have you gone back and actually looked at subclones from the PCR products that you amplified to see if those are, in fact, reasonably monomorphic as compared to what you had seen in mouse DNA?

DR. LO:

I do not really understand your question. You say, if we go back to --

DR. COFFIN:

When we amplified products that we attribute to contaminating mouse DNA, what we found was that in many cases the amplification gave rise to mixtures of products, and so you needed to do further dilution or subcloning of the product to actually get clean sequences. But if you just take the sequence that is fed back to you, you won't notice that until you actually subclone all of the mixtures of DNA that might be in that. Have you done that?

DR. LO:

For most of the sequences, we directly read -- and when we subcloned it, of course, the majority of the sequences are exactly the same that we directly read. We do have, for example, 10 percent, 15 percent -- they will have a mutation or replacement of nucleotide here and there. Essentially, the -- DR.

COFFIN:

Have you done that with all of them? Whether the band is monomorphic or not will depend exactly on the quantity that you start with. DR. LO: The majority of those sequences -- the predominant sequence is the one we actually read. We also, following your suggestion, will go back to do the single-gene amplification. Essentially, the majority of the sequences are consistent with what we have.

DR. HOLLINGER:

I just want to ask one question. You said you had eight donors that were positive in your assay, and then you recalled those donors -

DR. LO:

No, no. Previously, in the mid-1990s, those 37 patients tested positive, and 25 of those patients originally came from Dr. Komaroff's clinic. He went back to trace eight of those patients.

DR. HOLLINGER:

These did not come from blood donors?

DR. LO:

These were not blood donors. These are the patients with CFS.

DR. HOLLINGER:

These were not blood donors that you looked at later.

DR. LO:

That's correct.

DR. HOLLINGER:

So they have not been looked at over a period of time.

DR. LO:

Right.

DR. HOLLINGER:

Dr. Klimas.

DR. KLIMAS:

Two questions.

The eight follow-up subjects, do you know if they were more or less ill than they were the first time?

DR. LO:

Actually, I think Dr. Komaroff should be the one to answer this question. I asked him, and he said those patients are still sick. There is a variation among those patients, but most of those patients continue to be sick over this period of time.

DR. KLIMAS:

The samples that were in the freezer all these many years, had they been manipulated -- frozen/thawed or in any way -

DR. LO:

No. Regional samples in this study have been kept frozen for all this period of time. They have never been thawed out to use for different studies.

DR. GLYNN:

I just have a question for John, if you don't mind. If there was contamination, how do you explain the differential between patients with chronic fatigue syndrome and the normal donors?

DR. COFFIN:

I would have a lot of trouble explaining that if the samples have been taken contemporaneously and handled identically and blinded. In the case of the studies that I am involved in as well -- and it's the simplest thing to do, starting out -- the samples from the patients and from the donors were taken completely separately, at different times, different places.

I'll share with you my nightmare. That is that somewhere there is a warehouse that has a big pile of sodium phosphate sitting there. At night, mice come in and crawl over this and do whatever mice do. Then during the day somebody comes in with a shovel and grabs a few kilos of this, and that ends up being you reagent-grade PBS. That sometimes happens and sometimes doesn't.

The sensitivity of these assays is good -- a 100th of a cell is enough to -- this is the kind of thing we have to be extremely vigilant about in these kinds of assays, where you are just taking the sequence, where you don't detect a virus. I'm not saying that Dr. Lo is wrong on this. I'm just saying that the standard of proof has to be extraordinarily high.

DR. LO:

I totally agree.

DR. COFFIN:

I'm not completely convinced that the mitochondrial DNA is -

DR. LO:

I totally agree. In this kind of assay, obviously we have to be extremely careful. I just want to say, yes, we did pay attention to that. Every time we run all these assays, we put it in enough negative results and run it together. We have to say, in our system, we have never seen that.

DR. HOLLINGER:

Dr. Nakhasi.

DR. NAKHASI:

In the second group of patients that you recently got from Dr. Komaroff, seven or eight people, did you attempt to isolate the virus? I know you told us earlier that you could not from the frozen samples. Was there any attempt to isolate the virus from these samples?

DR. LO:

We did that. We obviously tried very hard to isolate the virus, because that will be the key question. When we have the samples, we set up all kinds of assays and tried to isolate virus.

At the present time, I have to say that we have not successfully isolated virus. We did see some infections in the signal, documented by PCR, but we could not have the virus isolated.

DR. HOLLINGER:

One comment, and then we're going to move on.

DR. ALTER:

I just want to perhaps clarify something. In terms of contamination, Shyh has covered most of it. To me, it was very important to get these eight samples from 15 years ago. These were drawn in the clinic, sent directly to Dr. Lo, and analyzed immediately. If a contamination was coming from a reagent, these were samples drawn differently, the sequences had changed over time, and the controls were again negative. It just doesn't speak to me of a contamination. It's not just the original two samples, the donors coming from one place and the patients coming from another place. This is a third sampling now from the same patients, drawn and tested immediately, leaving less room for contamination. You can't rule it out.

But I also wanted to ask Shyh, didn't you use Dr. Coffin's method on these samples as well?

DR. LO:

What do you mean, Dr. Coffin's method?

DR. ALTER:

For the IPA for the mouse contamination.

DR. LO:

Yes.
We checked the IPA.

As I described, in our hands, the sensitivity of the IAP assay to amplify lots of a side band from the human sequence and the sensitivity when we compare with the mitochondria PCR the same mitochondria PCR for the same set of human DNA -- with a spike in mouse DNA, we certainly see that the mitochondria PCR assay, in our hands, is much more sensitive. That's all we can say.

DR. ALTER:

But did you test our samples by that method?

DR. LO:

Right. That sensitivity was much lower, and we could not really document the IAP sequence there.

DR. HOLLINGER:

Thank you, Dr. Lo.

Agenda Item: U.S. Study

We will move on to the next U.S. study, Maureen Hanson, from Cornell.

DR. HANSON:

Thank you.

I am going to be calling this study the 20/20 study. It's a small study, funded by an exploratory grant from the NIH, an R21. This study is entirely unpublished. It's still in progress. We haven't been able to complete all the tests at this point.

I would like to start with acknowledging my collaborators. In my own lab, I have a postdoc, Li Ling Lee, and a part-time technician on this project. We have been assisted by David Ruppert, a statistician. The clinician who has provided the samples is David Bell. He has been assisted by his son, David Bell.

Forty subjects were selected by Dr. Bell. Dr. Bell presided over one of the outbreaks of CFS that occurred in the mid-1980s in his area of western New York. We have 10 what we call severe CFS, 10 recovered CFS, and 20 healthy controls. The Bells administered a number of survey instruments to test the health status of these healthy controls and the severe and recovered CFS. The samples were received at Cornell. We were blinded to the health status of the individuals providing the samples. I had to be unblinded in September in order to present -- in August, actually -- to present the talk at the XMRV meeting at NIH. But my lab members are still blinded because the study is not yet finished.

I would like to describe the very severe CFS. These people are very ill. They are housebound. They are often bedbound. They have less than three hours daily of upright activity. The recovered CFS all met the CDC definition of chronic fatigue syndrome at one time, but they all consider themselves either recovered or nearly recovered. They have many more hours of upright activity, 13.5. Six of these 10 were part of the outbreak that occurred in Lyndonville, New York. Of the healthy controls, all of them live in the same geographic area, western New York. They were screened so that they had never lived with a person with CFS, fibromyalgia, or prostate cancer. However, some of these people are close friends of CFS patients. These people have 15.5 hours of upright activity, and they appear to be completely healthy.

If we graph the scores on the survey instruments, you can see that the blue here, the severe CFS patients, have significantly different scores than the healthy controls. But what's interesting is that the recovered patients, although they consider themselves recovered and perfectly healthy, actually have some lower scores on many of these instruments, significantly lower on the SF-36 test.

These people do feel well. They feel well enough to donate blood, and a number of them, in fact, have done so.

We are still working out our assays for looking for the MLV-like viruses. We have a number of assays that we have tried, and I'm going to mention these. But we're still not sure which are the best ones to use. We have results from all the different assays, but we are still, as I said, in progress.

We have made PBMCs from blood collected in EDTA and made nucleic acids from these PMBCs that are immediately frozen or cultured for 5 to 10 days. We make RNA with TRIzol and make cDNA. We make DNA with CTAB. Then we do PCR on these samples. We observe DNA bands on the gel. We never just conclude from the size of the band that we have a gag sequence. We always sequence it before we conclude that we have a positive gag sequence. We have also recently been doing PCR assays on whole blood. We are using the Qiagen kit -- again, making DNA, doing PCR, observing the bands in the gel, and sequencing.

We have also been looking for virus in plasma by incubating it with the prostate cancer cell line LNCaP cells. Again, we make RNA, convert it to cDNA, make genomic DNA, and do PCR.

We do take precautions against contamination. We have been using mouse mitochondrial DNA controls, using cox2 primers. We are actually thinking of switching to the ones that Lo described, because he has validated how sensitive those are. We also do all of our work in labs that have never had any sort of mouse cell line or mouse work done in the lab. In fact, we put our hood into a plant growth room. All of our blood is collected in this former plant growth room. We UV-irradiate all of our tubes before we use them. We carry out our first reaction PCR in one of these hoods that has a UV light in it to clean up any DNA that may be in there. We have a second hood for our second PCR. This is all in a lab that's separate from my lab, where we work with the amplicons.

This is just an example of some of our results. This is actually genomic DNA from these LNCaP cells, incubated with plasma and then assayed after four transfers. We have not had very good luck in any of our assays with just a single round of PCR. Usually you get a gel like this, which is blank. But in second-round PCR, we have been able to detect gag sequences. This is after six transfers. These are different samples. They show again that we can detect gag when we sequence this. We often, like Dr. Lo described, get nonspecific human DNA bands of different sizes. Whenever we sequence a band that is the wrong size, it turns out to be some accidental amplification of human sequences.

Our sequences that we have obtained are very much like the ones described by Dr. Lo and the Alter group. We, in fact, have one of their blood donors up here. In comparison to XMRV, our sequences look a lot more like polytropic MLV. One thing in particular is this deletion right here in XMRV in comparison to polytropic viruses. We don't have that deletion, nor does the Lo, Alter paper either. This deletion was first described in the prostate cancer study, present in the glyco-gag region of XMRV.

This is a summary of our current data. We have 7 out of 10 severe CFS patients, 7 out of 10 recovered CFS patients, and 4 out of 20 healthy controls. These are only the numbers where we have done two different tests and gotten positives. We actually have some additional samples that are positive, but we only did it once, and so I'm not reporting that here at this talk. You notice that we have rather a high healthy control number here. It's my suspicion that this may reflect the fact that the samples were taken from an outbreak area, and although these people weren't living with people with CFS, as I said, many of them were friends and associates of people with CFS.

Why are there so many reports of failure to detect these viruses? One thing I would like to point out is that some of the negative reports -- the people used primers that span this deletion. A primer that spans this deletion is not going to amplify the virus that either Dr. Lo reported or that we are reporting here. This primer will not work. But it was used in at least two of the negative studies. The other possibility is that all of the labs with negative reports optimized their PCR conditions for detecting VP62 XMRV or XMRV from a culture and they didn't optimize for any MLV-like virus that might be present. In my lab we haven't had any XMRV in the lab. We have just optimized according to what's in the samples. We have tried lots of different conditions to figure out good conditions to use to look for virus in the samples, not using XMRV as our control.

For example, just to illustrate what different results you can get with different conditions, here's a case where we have four samples, and these two samples are negative under these conditions and these two are positive. But using these conditions, we get all four of these samples positive -- these, incidentally, being the conditions that were used in the Lombardi et al. paper. The other thing that we have noticed is that really very small differences in your methods can make a huge difference in the results. We have three different brands of PCR machines in our lab. We have seen differences in which PCR machine is used, in our own lab, as to what results we get as far as detecting things or not detecting things.

I would like to end by presenting some results that were not -- these data were not obtained in my lab. These were obtained by Rachel Bagni's group at NIH Frederick and at the WPI. Samples were sent from Dr. Bell to these groups to look for antibodies in the sera of these same people. Of the seven severe CFS samples in which we detected virus, at NIH six were detected as having at least one XMRV-reacting antibody. The WPI detected the seventh.

Clearly the NIH and the WPI tests are a bit different. You are going to be hearing about what those tests are later on in this session. Of the seven recovered CFS, NIH detected four of them as having an antibody to at least one of these antigens, and WPI detected the other three. Of the four controls -- this was quite reassuring to us -- in which we detected virus, NIH detected three as having one antigen and WPI also detected one of those three.
I would just like to mention that, of course, antibodies are less specific than PCR, and an antibody that reacts to XMRV should also be likely to be reacting with an MLV-like virus.

Our conclusions are:

- We have recovered patients who feel well, but they are actually significantly different from healthy controls.
- We have gag sequences similar to polytropic MLV.
- We have been able to infect LNCaP cells with patient plasma and get gag sequences after four or six transfers.
- Our virus sequences are highly similar to those reported in the PNAS paper by Lo et al.

Thank you.

DR. HOLLINGER:

Thank, Dr. Hanson.

Questions for Dr. Hanson from the committee? Dr. Coffin?

DR. COFFIN:

You do not have a virus that goes with these sequences at this point. Is that correct?

DR. HANSON:

We only have gag sequences at this point. We don't yet have the virus.

DR. COFFIN:

I will ask another one of the same questions I asked him. The sequences that you report, are those based on bulk sequencing of bands or are they based on cloning or limiting dilution of PCR?

DR. HANSON:

These are based on bulk sequencing. However, we don't merely just take the sequence that the facility gives you. In addition to getting the actual text sequence, we also get the actual traces. We know from our other projects in the lab -- my lab works on RNA editing as well -- that if there is about 15 percent of a different sequence, we can see it just in the bulk sequences.

So in some of our bulk sequences, we can see some single-nucleotide polymorphisms. Sometimes we can see a G in the provirus and see a G and an A in the cDNA. We can see that. I think if we had more than 15 percent of a different sequence, we would actually be able to see it in the bulk sequences.

DR. COFFIN:

But you do see some.

DR. HANSON:

We do see some, but single-nucleotide polymorphisms, not --

We are at the limits of detection. We will have a sample positive one time and then negative another and then positive the third time. The PCR is really tricky. As I said, sometimes when we got inconsistent results, we discovered that one person was using one PCR machine and another person was using the other. So you really are at the limits of detection with the PCR. That's why we want to get at least two positive results before we count a sample as positive.

DR. HOLLINGER:

So basically they were tested multiple times?

DR. HANSON:

Yes.

DR. HOLLINGER:

Dr. Nelson?

DR. NELSON:

I was just thinking of the causative criteria, the Koch hypothesis, et cetera. You had a group who had severe disease and another one who had recovered and then another one that was healthy. I guess this is totally qualitative data that you have. But I would just wonder, is there any way to quantitate whether or not the signal you get is different. Does that correlate with the then, obviously, the other criterion is temporality. In order to establish a cause, the exposure has to come before the outcome. I just wonder if there is any way to get at that, in terms of the age of the patients or when symptoms occurred or the age of the controls versus the cases. Can you see any way to look at these two criteria namely, temporality, which I think is the most important, and a quantitative relationship?

DR. HANSON:

First of all, as far as characterizing the patients, we were quantitative in giving them survey instruments, so we do know their health status. Many of these patients have

DR. NELSON: But what about the virus?

DR. HANSON:

Many of these patients have been ill for 25 years. Many of even the recovered people got sick in that 1984-to-1987 outbreak and then they recovered, most of them five or six years later. As far as the quantitative PCR assay, we are not there yet. I know there are groups, represented here, trying to develop a quantitative PCR assay. We don't have a quantitative assay for the virus at this point.

DR. KLIMAS:

Congratulations on such a nice study. It's really well done.

Your question is a very good one. The idea of using this epidemic group and comparing it to a cross-section of everything else that is out there would be very interesting. I think that would be good. I think it's very interesting that these so-called recovered patients are equal to the ill patients in the numbers you are calling positive.

At the XMRV meeting, I came away thinking that the LNCaP cell line, the prostate cancer cell line, wasn't as good for MLV as it was for XMRV. Did I hear that wrong?

DR. HANSON:

That is the conventional wisdom. We have actually never seen what is called classic XMRV. We have only gotten these polytropic-like sequences from the LNCaP cells. I can't explain that at this point, but, in fact, what we are seeing is polytropic-like virus in the LNCaP cells.

Let me also say one thing about epidemic versus non-epidemic. Not all of these individuals were people from the outbreak. There were people who you would call sporadic cases that appeared since then. There are some more recent people mixed in with the severe and the recovered. It wasn't just the 1985 outbreak.

DR. KLIMAS:

Did they scatter into the positives?

DR. HANSON:

With the small numbers that we have, I don't think we would have any statistical power to tell you that.

DR. HOLLINGER:

Thank you very much.

Agenda Item:
U.K. study

We'll move to the third talk in this section. It's a U.K. study. Dr. Mikovits will present this.

DR. MIKOVITS:

Thank you. Today I have been asked to talk about a study we presented at the XMRV workshop for a group of patients in the U.K. Since the first isolation of XMRV from the blood of CFS patients, my collaborators and I at the NCI, SAIC, and Whittemore Peterson Institute have been working to develop more sensitive assays for detection of infectious virus. The rationale for these studies is that in our work over the past year we have developed more sensitive methods for both the biological and molecular amplification of human MLV-related viruses, which in this talk will be called HMRV, in the blood and plasma. We have developed these technologies. These methods were used to determine the incidence of HMRVs in a U.K. cohort of ME/CFS. Importantly, this cohort was diagnosed using the more rigorous Canadian Consensus Criteria. Those are the criteria that Tony Komaroff and the study you heard from Dr. Lo, David Bell, as well as Dan Peterson from the original Science paper, used throughout their patient populations. It's important, because of the heterogeneity of the disease that we heard from Dr. Hewlett earlier, that the cases were similar.

Let's talk a little bit more about the study cohort we used from the London area of the U.K. They had gotten diagnosis of what they call myalgic encephalomyelitis. Or, often, post-viral fatigue is how it's diagnosed in the U.K. primarily. All of these patients do meet the Canadian Consensus Criteria of CFS that are used in those studies that we talked about today and in our original study and throughout the WPI. The disease duration in this patient population was 9 to 26 years, with greater than 50 percent of the patients actually housebound, and many bedbound. The onset of disease could often occur in childhood or puberty. We won't go into the possible reasons for that today. In addition to that profound post-malaise/fatigue that is really the sine qua non diagnosis of CFS that meets the Canadian Consensus Criteria, the other symptoms included severe cognitive dysfunction, multi-joint pain, the onsets of new and frequent migraine headaches, vertigo, dizziness, lymphadenopathy, profound mitochondrial dysfunction, which might explain the energy. Many of these patients have GI disturbance and dysbiosis, an inability to absorb nutrients, and medications as well. They have chronic infections. As we have often heard, well, those CFS patients have everything. Yes, they do, and that's the point. A healthy immune system doesn't have chronic EBV or chronic HHV-6. We see shingles in a 30-year-old often in these patient populations.

Importantly, many report a flu-like onset. They knew the day they got sick, and didn't recover. The current age of the study participants that were used was 19 to 70. Interestingly, in the 50 that were done randomly in this first pilot study, equal numbers were male and female. The study design we used is similar to that which we used in other studies done at the WPI. We have the blood drawn by Phlebotomy Services International, which is an independent certified phlebotomist group that goes around the world. PSI codes and ships those samples. In this case they were shipped to the NCI, where they were processed in a laboratory that had no previous XMRV work nor any previous murine research -- a human lab that had not done XMRV research previously. The plasma and the PBMCs were isolated two days after the blood collection. That was largely a matter of the shipping of the samples from the U.K. All the samples were tested in two independent labs, blinded. We blinded in 50 healthy controls taken from blood donors by our collaborator, Jonathan Kerr in London, in the mid-2000s, 2005 to 2008. We didn't have fresh draws from those blood donors, but these were blinded into the study, as those were the controls that we had available.

The samples were tested for the four methods. I'll go through it very carefully:

- For plasma XMRV RNA.
- For cell-free transmission from the plasma to the LNCaP cells, which we have heard about earlier. I'll describe that assay in detail.
- We looked for plasma antibodies to HMRV viral proteins.
- We Western-confirmed the positive cases from those transmission studies.
- Finally, we did sequence characterization of the HMRV isolates.

First, we'll talk about the plasma PCR. We had never before done direct plasma PCR. We had been working with the Blood Working Group and thought that perhaps delayed processing, which had been done in other studies, might increase our ability to see viral RNA which may have been associated with other blood components and actually released into the plasma. When we did this plasma RNA from 140 µL of the plasma from 48 percent or 24 percent of the patients, we could see in this top, using the Lombardi nested primers and conditions, a very strong band for the gag. These were all sequence-confirmed to be gag of XMRV, but this amplicon wouldn't distinguish the polytropic sequences. It was very small.

Secondly, we used the Lo primers, went back to these samples that had not been previously frozen and thawed, because we aliquot them into .5 mL aliquots. You can see that two patients who were negative using our PCR were positive using the PCR protocol of Dr. Lo, suggesting that that also sequenced. But we didn't see it as polytropic. Maybe that's just our phylo-genetic analysis. But all were confirmed by sequences and again highlight that subtle differences in PCR protocols can give you really big differences in results, as you would have found far fewer of these patients positive by the Lo protocol. Importantly -- and, unfortunately, it doesn't show well here -- we used 5 femtograms of DNA from the murine cell line that we have in the lab.

We could see no mitochondria-specific amplicon, as Dr. Lo described.

The other thing that we do in all of our studies you see here number 2767 -- those are patients from the original WPI Science study, where we consistently and over time -- over three years' time -- can both detect plasma viremia and isolate from that patient. We carry this sample throughout these studies. We do that with several samples in every study. The control samples, as I mentioned, that we blinded in from Dr. Kerr -- you can see that very few actually had XMRV or HMRV RNA in the plasma. But importantly, two out of 50 that were reportedly from the healthy blood donors -- was 4 percent of the population there in the U.K. I do want to remark that of the two negative studies that had come out at this time from the U.K., they had absolutely zero incidence in controls or patient population of XMRV.

Since we are not a PCR lab and neither we nor the Science paper nor our work focuses on PCR, we went to doing the culture techniques that you have heard about today. I'll describe them in a bit more detail to show the isolation and characterization -- that these were indeed representing infectious virus. In the assay that we used in the Lombardi study, shown in the top line here, we take plasma or activated -- this is dividing peripheral blood and mononuclear cells -- from the patients, and we co-culture them on the prostate cancer cell line, which was responsive to androgens and inflammatory cytokines. This is important because we know we have characterized the LTR, in Steve Goff's lab and Bob Silverman's lab, and we know that there are hormone-responsive elements there that would be an on switch to make the virus replicate more in the cells that were responsive to androgens.

We culture these for 21 to 42 days. Dr. Hanson mentioned four passages. This is a lot of cultures. Carefully looking at other negative studies, they might culture them for a week. We follow them, in addition, to PCR by Western blots from antibodies. These are monoclonal antibodies which were described in the original Science paper. This rat monoclonal to the envelope of this spleen focus-forming virus, which is a polytropic, xenotropic virus -- importantly, this antibody was characterized by Sandy Ruscetti all the way back in 1982. But this surface unit I show you here in the Western for the transmission of three of these U.K. patients -- this antibody recognizes all polytropic, xenotropic, and ecotropic viruses. This antibody -- and that may be why our numbers were so high, because our original paper didn't originally rely on just the PCR, when, in fact, this antibody could detect all of the viruses. I'll refer you to that paper. If you look at Figure 1, you will see PCR-negative patients who clearly we could culture virus in, detect it from the antibody, and sequence whole virus.

This is the assay, which is quite labor-intensive and cumbersome. You know that it took us quite a while to do these 50-odd samples and 50 controls. We have been developing -- and you heard this from Dr. Le Grice at the last BPAC meeting in July, so I won't go into detail -- an assay in which an MLV vector has an inactivated green fluorescent protein in it. That vector is packaged by either XMRV or any MLV-related virus.

You then infect those cells with that virus, if it's in the plasma, and in only 4 to 18 days, you can see green cells, representing infectious virus. In order for this vector to go from inactive to activated, it needs both reverse transcriptase and integrase. So it's important that this assay is an assay for infectious virus.

We show here that you can also quantitate it by flow cytometry and clearly see and count the green cells. Hopefully, that has been speeding things up a lot. When we use it in the U.K. samples, here is a positive control. Only 11 percent are positive. But that is due to viral interference and other things about this assay. But clearly a negative and clearly a positive. Both of these samples, if you go back and look at that first figure, were plasma PCR-positive, suggesting, but not proving, that it's infectious virus. Now we can see that 78 percent, 39 out of the 50, were positive in this infectious assay. This is just showing you other numbers that were negative and positive in the same assay, as you can see here. When we confirmed all of the samples -- and I show you here only the positive -- we confirmed by Western analysis, using an anti-MVL envelope. This is a xeno, so it is not the monoclonal I showed you, and then a Gag antibody as well. You can see that we can detect both Gag proteins and envelope proteins in these Western analyses, confirming that we had, in fact, transmitted the virus from the plasma of these patients to the LNCaP -- we call these DERSE cells.

Importantly, I show you that 2767 positive control that we carry throughout these studies. We next amplified a wider range, shown in the box on the top, of the envelope. When you look at small amounts of envelope, maybe due to the diversity that is wider than we originally anticipated -- when we actually did a PCR in the pol-pro region, extending down 600 base pairs of product into envelope, and then we sequenced -- I show you here, representative, three of these U.K. samples we could see that they were indeed more similar to XMRV than to the polytropic viruses we have been hearing about this morning.

Interestingly, this patient, U.K. 1023, was negative in all of the other assays, but we could actually, from the LNCaP, which was a DERSE assay -- it was only 2 percent, but we could actually see by Western that there was indeed virus there, and we could clone it out. We are doing full-length sequencing of as many of these viruses as we can from single cells at this time.

We next talk about the serology assay in these patients. This is the assay that you heard from Maureen Hanson and that was described in detail in the Science paper. We use a cell line that expresses the murine spleen focus-forming virus envelope, that same region that I showed you. The antibody recognized all known polytropic, xenotropic, and ecotropic viruses. We take a plasma from a patient sample and incubate it with the non-expressing cell line. You see nothing in this histogram, which shows increasing fluorescence and density of the binding of a secondary fluorescently labeled IgG. But here, with the patient sample on the envelope-expressing cell line -- clearly suggested that there is antibody to the envelope in the patient's plasma.

You can compete that using that monoclonal antibody. If we co-culture the monoclonal antibody with the patient sample, you see that you can compete either at 1-to-10 or 1-to-50, demonstrating the specificity of this antibody and that indeed the patient samples do contain antibodies to spleen focus-forming virus envelope.

This is how the shift looks. We did this in all of the patient samples and controls. When we compare the detection of antibody reactivity with virus isolation from the plasma, you can see a concordance there in essentially all of them. There were only five samples where we could isolate virus, but could not detect presence of an antibody -- we don't know why that is -- and a few samples where we could detect antibody and not actually isolate virus.

In summary, then:

- We could detect gag in the RNA in the plasma in 58 percent of the 50 patients.
- We could transmit 78 percent of the patient samples to the LNCaP cells.
- We see antibody reactivity in 68 percent of those 50 patients.
- We could sequence the envelope products, showing that the predominant HMRV in this U.K. cohort is indeed XMRV.

We conclude that multiple methods are necessary to detect evidence of XMRV infection. In this case, in a very well-defined cohort, similar to the positive studies, we could detect it in greater than 70 percent.

With that, I'll thank my collaborators and funding and you for your attention.

DR. HOLLINGER:

Thank you.

Questions?

DR. COFFIN:

The virus is growing out in the DERSE cells. How much of that have you sequenced?

DR. MIKOVITS:

We have sequenced large parts of the envelope and the gag. We -

DR. COFFIN:

I'm just curious, because one expectation in those cells is that what might happen -- the virus that grows out may not actually be the virus that you originally started with.

DR. MIKOVITS:

That's why we –

DR. COFFIN:

It could be a recombinant that has picked up useful sequences from the vector, like the LTR. It would be nice to see if that's happening.

DR. MIKOVITS:

Indeed, and that is why we also run those LNCaPs without that vector. We have been sequencing the virus out of the LNCaPs where I showed you the Westerns. We run both assays, because we recognize that that might happen. It would be interesting if it did indeed happen.

DR. RUSCETTI:

Can I follow up on the Coffin question?

DR. HOLLINGER:

Could you give your name?

DR. RUSCETTI:

Ruscetti, NCI.

We have done LNCaP and the DERSE cell on several isolates and found no difference, at least in the envelope and gag regions.

DR. COFFIN:

I was thinking particularly about the LTR, which might well have been exchanged.

DR. RUSCETTI:

We are just beginning to work to look at the LTR. We have been pushed into it by Jonathan, who asked us at every meeting to look at it. So we are now looking at it. But we don't have any results for it.

DR. MIKOVITS:

And it could well be a key to the reservoir. The LTR is really a key, maybe, why we can't grow this virus in these cells. We are looking at other cell types right now.

DR. COFFIN:

That harks back, in a sense, to the other talks. To my knowledge, nobody has ever grown a virus that has a polytropic or a modified polytropic-type LTR in it, unless somebody in the room has done that recently and I haven't heard about it. DR. MIKOVITS: Usually these are seen with xenotropic, actually mobilizing the polytropic. The polytropic indeed becomes the pathogenesis. But they need the xenotropic to be --

DR. COFFIN:

In mice that seems to be the case.

DR. NELSON:

These are pretty convincing data, to me, that this infection is real. You showed antibodies. One issue that remains is, is this the cause or is this the result? It's quite possible that they have something else - the immune system crashes or whatever -- and they get infected with whatever is around, a mouse or whatever. Have these patients been studied for other chronic viral infections, HHV-6, HTLV-2, or EBV? Have these patients had an extensive virologic/infectious disease workup?

DR. MIKOVITS:

These patients in the U.K. have not. It is a psychosomatic disease in the U.K., and they can't get those types of medical treatments easily and maintain their benefits. In our study in Science, the answer is yes. These patients have multiple chronic active infections -- EBV, HHV-6, CMV, as I mentioned, shingles. We see everything -- mycoplasma. It looks to us like an AIDS patient, with an obvious hypothesis being that the retrovirus causes the underlying immune deficiency. But it alone can't cause the disease. It needs the co-pathogens. You can have HIV without having AIDS, but you can't AIDS without having HIV and one of 25-odd co-pathogens.

DR. NELSON:

So it looks like the next step is to there are several repositories, and this disease may be frequent enough that one could identify an infection with this agent. You have 4 percent of blood donors or whatever. These people need to be followed. These people need to be followed to answer the question: Does this infection occur before the chronic fatigue syndrome or afterwards? If it occurs afterwards, then it's just a passenger; it isn't the case. I think that's a critical question.

There are some repositories, the NHANES, Washington County, Maryland, where there are large numbers of samples that are stored and frozen, to go back and look at incident disease after that. It seems like this should be of some priority at this point.

DR. MIKOVITS:

It is a critical question, but we can't do it with the existing assays. Part of the reason we were developing these, hopefully, more high-throughput assays is so that we could do the large-scale epidemiological studies necessary. I agree completely.

DR. HOLLINGER:

Dr. Coffin, one more.

DR. COFFIN:

In that same vein, it's also not out of the question that this virus infects very large numbers of people and remains in some very-difficult-to-detect form, as it does, apparently, in macaques after they are infected, as we'll see, and that there is something about a condition like chronic fatigue that allows it to appear and replicate in that fraction of people, despite the fact that it's in almost everybody to begin with, or in a very large fraction of people to begin with.

DR. MIKOVITS:

If it were, we might find it a little more easily than we do.

DR. COFFIN:

We might, but it's not so easy to find Epstein-Barr virus if you don't have an antibody response either in infected people. It's very, very infrequent in latent infection, but it's there. It might be that there is -- I think it's a remote possibility, but a possibility that in some people there is some hidden reservoir somewhere that only becomes visible due to some condition associated -- immune deficiency or whatever -- associated with chronic fatigue.

DR. HOLLINGER:

Thank you, Dr. Mikovits.

Now we'll go back to the one we were going to start with. Dr. Jonathan Stoye is going to talk to us about a "Summary of the Current Research on MLV-Related Human Retroviruses and Disease Association."

Agenda Item:
Summary of Current Research on MLV-Related Human Retroviruses and Disease Association

DR. STOYE:

I thought I was giving the introduction. I'm not. Some of my slides may be repetitious. I apologize for that, but there's not much I could do about it.

It is, actually, rather better than something that happened one time I was in the States before, where I had flown over for a day to give a talk on a certain topic and found myself pushed into a discussion session. It got later and later and later in the day. It was about 6:00 or 7:00 at night, U.S. time, and it was well past midnight my time. This time at least I'm awake.

So I'm going to give you a brief introduction. I want to say that I am the head of the Division of Virology at the MRC's National Institute for Medical Research. I have worked with murine leukemia viruses for longer than I care to remember, certainly more than half my life. What I was going to do was summarize recent data on the detection of murine leukemia viruses in human samples and describe their association with disease. Then I was going to consider and present my perspective on the data that I have described. I think this is probably still relevant to you, even though you will have heard at least some of it before.

This field started in 2006, with a paper from Bob Silverman and his collaborators, who isolated a virus they called XMRV from prostate cancers. They found it in around 10 percent of prostate cancer tumors, associated with a deficiency in an immune defense gene. Since it was similar by sequence, they called it XMRV.

This stimulated a number of different studies that I'll tell you about in a few minutes, but first I want to say a little bit about some of these strange words -- "gammaretroviruses," "xenotropic" -- just to make certain you have been listening. I will test you afterwards to see whether you know what they mean. This slide shows a tree of retroviral phylogeny. There are seven genera of retroviruses. You are familiar with HIV, which falls in the lentivirus genus, or HTLV-1, which is in the deltaretrovirus genus, and XMRV falls, with the murine leukemia viruses, within the gammaretroviruses.

These gammaretroviruses are simple retroviruses. They don't have the accessory genes that are present in some of the viruses, like HIV or HTLV-1. They are widespread in nature. They have been isolated from many mammals and koalas. Infection is lifelong, with persistence of infected cells established very soon after infection. They have been associated with a large number of diseases, frequently with cancers.

These viruses can give rise to what are called endogenous retroviruses. These are retroviruses that could infect the germline and are present in the germline of essentially all mammalian species.

Different endogenous retroviruses are present in different numbers within these groups, and it's perhaps not well appreciated that, in fact, we have more viral sequence in us than we have coding genes. So we are more virus than human.

The first recognizable endogenous retroviruses were inserted more than 50 million years ago. So back in the age of dinosaurs, retroviruses were first floating around the world infecting our precursors. These viruses have, on the whole, suffered from various different inactivating mutations that don't give rise to virus. Basically, we have lived in a sea of retroviruses for more than 50 million years, and there has been constant conflict between retroviruses and us.

We have developed various ways of trying to protect against these things. Very few of them, in fact, can give rise to infectious virus. So the defense mechanisms have succeeded in inactivating these long enough for random mutations to have occurred. They can give rise to infectious virus occasionally or can contribute sequences to replicating viruses to change their properties.

Xenotropic viruses were originally defined on their ability to replicate only in species other than mice, due to polymorphisms in the receptor gene. This idea may, in fact, have to change slightly. Recently it has been shown that certain mice do contain functional receptors for xenotropic virus. But it's likely that this name, as a label for a class of sequences inherited as endogenous forms, will persist.

Some of these proviruses -- for instance, Bxv1 -- are intact and infectious and frequently have been shown to infect human tumor cell lines passaged through nude mice. None of these xenotropic viruses is directly pathogenic in mice, but often recombine and contribute to the generation of oncogenic recombinant viruses. They can cause a wide variety of diseases.
The pathogenicity in other species is unknown. And they are closely related to XMRV, but none is identical.

Let me start the meat of this talk by presenting the association between these viruses and prostate cancer. There have been 11 published, peer-reviewed studies dealing with different cohorts of patients that have been examined for the presence of XMRV. There seem to be basically some groups that find around about 20 to 25 percent positive for XMRV. They constitute about a third of these. In the rest of them, there are zero or between zero and 1 percent detection rates. So you have these two schools: You either find it or you don't find it. That seems to be true for CFS in much the same way as it's true for prostate cancer.

Some studies show an association with RNase L, but, in fact, it doesn't look as though that association is holding up. In one study, with a relatively small set of samples, they were analyzed by three different techniques simultaneously. Five of these seven were positive by all three techniques. By contrast, two were negative by all three.

However, in the largest study showing an association between XMRV and prostate cancer, a number of samples were positive by one technique, but not by another technique. This is very worrying. One has to be concerned about the techniques that are being used to detect the virus. There are now half a dozen different techniques that are being used to detect virus. If you get a result with one but not another, one does have to worry.

This just compares one recent negative study, one recent positive study. Both used very similar techniques for detecting virus. One used a qPCR for envelope, the other a nested PCR for envelope. They worked with similar DNA. They have similar sensitivities. But one had zero out of 161 positive for XMRV; the other, 32 out of 144. Interestingly, the group that found positives, if they dropped the amount of input DNA for their PCR reactions, also detected a much lower frequency of DNAs, suggesting very strongly that it's very tough to detect this virus and you have to push your PCRs in order to find XMRV.

Which brings me to one of the questions I would ask: If the limit of detection is around one copy per 1,000 cells, one has to come up with plausible disease mechanisms if one is to invoke these as causative agents for disease. The first of the two studies on the previous slide, the one by Aloia et al., also used immunohistochemistry to try and detect the virus. They looked at nearly 600 samples. All were negative. They also state, but do not document, that they obtained negative results with sections from specimens found to be positive in the previous study.

Again, were enough cells studied? If the methods used here were correct, then there must be something wrong with the other study.

A key point in this saga was the paper that came out from Lombardi and various people you have already heard from on the detection of a virus, XMRV, in 67 percent of patients with chronic fatigue syndrome and in 4 percent of controls. This virus was found by PCR, serology, and virus isolation, and appeared to be robust.

If this figure of 4 percent of control donors is correct, then we clearly have a problem, because this is higher than the number of HIV-infected individuals in the world. And if this virus can cause different pathologies, it's a real problem.

However, various people have tried to replicate that study. And here I have to confess to a conflict of interest. I'm an author on one of these papers. Nobody, other than that first paper, with the exception of the Lo paper, which I'll come to in a moment, has been able to replicate Lombardi. So neither the initial association with CFS or controls has been substantiated.

I'll mention two other groups of studies before I go back to CFS. There are a couple of positive papers -- well, one is a paper and one is a patent – claiming that XMRV is present in the respiratory tract of immunocompetent and immunocompromised individuals. Similarly, immunohistochemical studies of breast tumors suggested that a fraction of these also contained XMRV.

Unfortunately, these studies remain to be confirmed by other techniques. It will be interesting to see what comes out. These are other searches for XMRV in groups of individuals where you might have risk of transmission through blood or sexually transmitted. They include several groups of HIV-positive individuals, a group of Hep C individuals, and also, interestingly, given claims of an association between XMRV and autism, there is a negative autism study. In this case some of the patients were autistic children of CFS-positive mothers.

In all cases the assays look pretty good here. I'm surprised that they are zero, if virus is really present in the number of individuals for which it has been claimed.

Let me go to the Lo paper. As you have heard, they detected MLV-related sequences in 32 out of 37 patients and 3 out of 48 normals. These were most similar, by sequence identity, not to XMRV, but other endogenous pro-viruses. And I must actually insist, since I coined this phrase, that what they are most similar to are modified polytropic viruses, not polytropic viruses.

They concluded that "our results clearly support the central argument by Lombardi et al. that MLV-related viruses are associated with CFS and are present in some blood donors."

I have concerns about that statement, for three independent reasons.

The first is that there is no virus isolation or evidence for viral insertion into human DNA.

The second is -- well, let me talk about the first one first. Let me describe the case of a virus that was at one point called HRV5, human retrovirus 5.

It was originally cloned by PCR techniques for a patient with Sjögren's syndrome. It was related to betaretroviruses of rodents. It was found in multiple labs, in various different conditions, and it was not found in controls. Independently, several different labs discovered this same virus.

Some patients had serum antibodies that reacted with HRV5 gag, but no virus was identified. When, finally, integration sites were cloned from clinical samples, they were shown to be rabbit, and not human. It's still not clear how multiple labs detected a rabbit endogenous virus in human tumor samples, but not in controls. But that's what happened.

To date, no infectious replication-competent viruses which are non-recombinant, belonging to the endogenous PMV class, have been isolated. We need urgently to see a more detailed characterization of the viruses described by Lo et al. In particular, I would like to see those eight new sequences which were reported two months ago, but we still haven't seen details of.

Let me talk about the question of variants. It has been suggested that xenotropic and polytropic viruses are simply variants, that they are more closely related than certain isolates of HIV-1. However, I would argue that that is not the case, for a couple of reasons.

Firstly, the four classes of endogenous virus that have been described in the mice have fairly defined and fixed properties, including a number of restriction sites and insertions shown here, but even at the nucleotide level. You can tell pretty much which one is which just by looking at a few hundred nucleotides of sequence. You don't see intermediates between these. They seem to be fairly fixed.

Certainly -- this is data from the recent review by Christine Kozak -- they evolved in different parts of the world, apparently to form a fairly stable form of virus. So xenotropic viruses and polytropic viruses came from different parts of the world.

I'm not convinced that these can be thought of as parts of a continuum. Rather, they are distinct viruses. Yes, they can recombine with one another, but they are not part of a continuum. So I would say these viruses are therefore unlikely to represent variants of XMRV.

If their results are correct, then it would imply that you have independent viruses, with independent transmission into the human population.
Lastly, I want to talk about a comparison of the sequence methodology used. This is a bit difficult. I have tried to do this a number of times with papers from a number of authors. Different primers have been used in PCR techniques, and different DNAs have been used. But there is a very small subset of the Lombardi paper in which they used the same first-round primers as the Lo paper -- so these 419F/1154R. This, remarkably, had a 50 percent incidence of positive results. This, I believe, was only 7 percent. This virus here was xenotropic virus; this virus here was a polytropic virus.

Interestingly, the primers used here -- this primer has mismatches with polytropic viruses. It's identical to XMRV. So if these samples had XMRV, one would have expected that this would amplify XMRV and not PMV.
So I have great difficulty in explaining how this could be. Based on these results, it is improbable -- highly improbable, in my opinion -- that these samples have XMRV. That result one can take further by considering the second-round nested PCRs, but I haven't got time to do that.

I would also compare the Lo data with that from another paper, by Henrich et al., which has just appeared. As far as I can make out, the technologies were similar, the primers were similar, the sensitivities were similar, the patients came from the same part of the world. Yet in one case you have negative, in another case you have positive data, both in the control and the CFS patients.

You have seen a version of this slide already. There have been a number of explanations suggested for these discrepant results -- differences in diagnostic criteria, differences in the geographic distribution of XMRV, PMV, and all the other ones, how the samples are processed, what sample was actually taken, the possibility of mutations introduced by APOBEC or other agents. But I don't think they cover all the cases all the time.

I think one gets very close to this problem, and the word that everyone wants to avoid using, which is "contamination." I might, in this context, change the word "improbable" to "unthinkable." But, nevertheless, I'm going to think it because of this problem.

I would stress that working at the limits of detection presents a significant risk of having problems. There are at least these five potential problems, any one of which might give you false positives.

The first is that you fail to sequence the products. Often you see human bands that contaminate gels. I'm not saying that these things have occurred multiple times, but I know of at least one occasion in the last year when each one of these has happened.

You can have amplification of endogenous retroviruses from contaminated mouse DNAs. You can have amplification of plasmid DNA or vector controls. You can have amplification of viral nucleic acids from "outer space." That means things that come up positive once, but not again. If you get a virus like XMRV, which will go through LNCaP cells like a rocket, I don't believe that this kind of thing can be -- that such sporadic isolation can be explained. I would argue very strongly that having positive controls, such as virus or plasmid, actually increases these risks. Unless you use a separate lab for each experiment -- you're going to run out of rooms in your building. I did wonder once whether you could just go down the corridor.

So I think there are real problems there. I'm going to conclude with three quick conclusion slides. There are various things we need, which include validated samples, better reagents, better case definition, and the results from the blinded split samples that I hope we'll learn about in the next few hours.

There are lots of things that I think we still don't know. We don't know where this virus came from, whether it crossed once or more. If the results are all correct, then it has to have multiple cross-species transmissions. We don't know about roles of disease. We don't know of incidence, distribution, mode of transmission.

So my take on all this is as follows. I don't think anyone would disagree with this first one: These levels are extremely low and hard to detect. I would argue that the samples studied by Lo et al. did not contain XMRV, and thus do not corroborate the data of Lombardi et al. There will be those who argue with that conclusion. However, if one wants to reconcile them, there have to be different transmission events, and if that is the case, then there have to be multiple origins for chronic fatigue syndrome. In other words, the samples that Lo et al. looked at have to be different from the ones from the WPI.

I think it remains unclear whether the XMRV presents any problem to the blood supply. I must say that the study that I was part of and other studies I know of in the U.K. have not seen the same results that we heard reported earlier.

I think I'll stop at that point.

DR. HOLLINGER:

Thank you, Dr. Stoye.

Questions? Dr. Klimas?

DR. KLIMAS:

I am left with just these big puzzles.

One is, why are the controls negative in the positive studies?

The other is, why is there antibody? It's not just a question of virus, contaminated virus, and so on.

The third is, my experience as an HIV doc for an awful lot of years -- I remember going to a conference where Dr. Ho announced that one in 10,000 cells were positive for HIV. The next year, it was one in 1,000, and the next year, it was one in 100. Finally, it got down to one in 10, over the course of just four years of improving assays and improving method.

So here we are with these assays that everyone agrees are just dreadful. Yet you can say with confidence how much virus is there? It doesn't make any sense.

DR. STOYE:

I think that these techniques are ultrasensitive, these PCR techniques. If there was one copy for 1,000 cells, you would find it -- not one in 100, not one in 10, but one in 1,000. You would find it. So that's the first thing.

The second thing is, precedent says there have been instances in the past where control populations were negative and others were positive. We don't know why that is. But that has happened before. So there is precedent for it. Certainly if the samples are not completely intermingled with one another, one does worry very much about somebody knowing what a positive is and what a negative is.

That does bring up a practical problem, in that I say one shouldn't have known positives in among negatives. I don't know what one does about that. What was the -- oh, the sera. I'm not a serologist, but my understanding is that you can sometimes get cross-reactivity. There have been cases where the antibody was directed against something different than you thought it was, even though it reacts with it. It has been known on more than one occasion.

DR. NELSON:

You showed a comparison of a couple of studies that used the same primers, the same methods. Have any of these tested the same specimens?

DR. STOYE:

I think we will hear about that in the study from the Blood Working Group.

DR. NELSON:

It could be that XMRV causes something one place and HHA-6 causes it somewhere else.

DR. STOYE:

I cannot rule that out. But I think we will hear in the next talk or two about studies on the same set of samples.

DR. COFFIN:

I would like to reemphasize a point that Jonathan touched on, and that is that our current information on the association of XMRV with disease is based on about five different things, including detection by FISH, for example, of small numbers of infected cells in prostate cancer, including isolation of XMRV from both prostate cancer and chronic fatigue syndrome, including detection by PCR of MLV-like sequences, including some serology and so on, and the detection by immunohistochemistry of probable viral antigens in prostate cancer.

I think it's very important at this point not to conflate those observations together. I often hear people talk about these as though they were all tips of the same iceberg. I think there is no reason to make that assumption right now. I think we have to consider each of these observations separately and independently.

As Jonathan eloquently pointed out, I don't think we can consider, for example, that the Lo et al. study confirms or refutes or really impacts the conclusions from the Lombardi study, because we are looking at two different things. Each of these has its own possible sources of possible problems, such as contamination, cross-reactivity, and things of that sort.

While I would very much like to have seen this subject go forward to understanding what the pathogenesis of the virus might be, we are still hung up, and we're hung up because we don't yet in any of these cases have the sort of convincing data that would reassure us that our concerns about cross-reactivity, contamination, and so on aren't true.

For example, if XMRV samples showed significant genetic variation within a patient and significant variation in the way that HIV does from patient to patient, we wouldn't be having this conversation. There wouldn't be an issue here. If there was a complete infectious virus that went with these MLV-like sequences, our conversation would be very different. But in none of these cases have there been -- although none of the experiments that have been done conclusively show that these, in fact, are due to these various kinds of artifactual situations, there is a lack of experimental evidence in any of these cases that reassures us that they are not. That's what is bothersome here. We need to keep moving forward and try to get evidence one way or another. But we are certainly not at that point yet.

DR. STOYE:

If I may just add one thing to that comment, there were 17 sequences that were described of envelope sequences in the Danielson paper. There were about 10 nucleotide differences in total between those 653 times 17 sequences. Now, by my calculations, assuming an error frequency per replication cycle at 10-5, that means that all those 17 cases had to be linked by less than a total of 100 replication cycles with one virus. It's, to me, implausible to see how that can occur.

DR. COFFIN:

But not quite impossible, unfortunately.

DR. STOYE:

By no means impossible, but implausible.

DR. RENTAS:

You mentioned at the end that one of the possible issues is the lack of validated positive samples. How would you define that?

DR. STOYE:

That is a very good question. You have to have a sample from a patient that multiple individuals can receive and all agree that this sample is undoubtedly positive. I don't think it's there yet.

DR. HOLLINGER:

Thank you, Dr. Stoye.

Let's move on. The next one is going to be about "Animal Studies: Potential Transfusion Transmission of MLV-Related Human Retroviruses," by Dr. Villinger.

Agenda Item:
Animal Studies: Potential Transfusion Transmission of MLV-related Human Retroviruses

DR. VILLINGER:

Thank you very much. Good afternoon. It's a tough act to follow, to say the least. My cohort is probably the only one that is smaller than Dr. Hanson's. But the price of monkeys is so high, on a weight basis.

Thank you again, for being here to share our findings in a nonhuman primate model, which, as you all realize, is fairly related to humans on the evolutionary scale.

I think we have heard enough of the background of the XMRV. It was discovered by Dr. Silverman.

What is important for us is really to keep an open mind. As I said, an etiological link has yet to be really established between XMRV and prostate carcinoma or chronic fatigue syndrome. However, I think one thing to keep in mind is that a number of these patients may suffer some level of immune impairment, and that may create a milieu that allows the virus to replicate. So I really think that in order to figure out what happens early on, since we see these patients relatively late in infection, an animal is where you can get some answers.

What we were trying to do was document whether the virus is able to replicate in a monkey model. Then, if it induced an active infection, what are the viral kinetics and behavior in vivo?

The primary question we had was, first of all, does it replicate in vitro in monkey cells? Number two, is there any preexisting immunity in rhesus macaques?

This was done right here, where we tried to replicate the virus in a primary fibroblast line that was developed in my lab. You can see that not only did the virus replicate, but we also got protein. This is an example of the animals that we have been using in the study. But we tested about 25 from the Yerkes cohort, and basically we didn't see any evidence of pre-existing immunity.

We enrolled five rhesus macaques. Initially we started with three, two males, of course, since we were interested in the prostate, and for the sake of balance, we had one female. We basically tried to stack the cards in our favor. We gave them a relatively high dose of XMRV intravenously, followed the animals, doing a number of different collections. Around five months post-infection, we sacrificed one animal and tried to reactivate the virus infection by reinfecting the two remaining animals IV with purified virus, followed them, and then ultimately, to boost the titers of antibody, we vaccinated them with recombinant XMRV proteins in incomplete Freund's adjuvant.

We necropsied the animals two weeks later. Then we went back, took two animals, and, once we figured out where the acute infection was, we sacrificed two animals during the acute infection stage.

This is the list of techniques we used to follow the animals. I will not show all the data, for the sake of time.

These are the viral loads that we observed in these animals. Basically, monkeys different just like humans, and we found three different viral load patterns. One animal had a relatively rapid peak of viremia, which was below 10,000 viral copies -- so fairly low -- and then was undetectable at day 14. The female animal had a delayed viremia, a lower one as well. In the third animal we basically never saw any viremia, plasma viral RNA in the blood.

However, when we looked at PBMCs, you can see that we were detecting proviral DNA in all three animals for about a month post-infection.

Reisolation of full-length RNA from these animals at these time points, day 18 and day 21 -- we were able to get full-length RNA, which was sequenced. Something that was brought up by Dr. Stoye in the previous talk is that there are a number of host restriction factors that will work on XMRV. It's a simple virus. It doesn't have all the accessory genes to counter these natural antiviral mechanisms.

This work was done in Bob Silverman's lab, looking at this one animal at day 21, analyzing about 1,200 bases. You can see that you have extensive mutations that occur, G-to-A hypermutation, which is probably the work of APOBEC3G, which is fairly active. That goes along with some of the questions that were brought about how wide you would find the same sequences maintained, especially in the blood.

The second monkey that we were looking at -- this is a female animal at day 18 -- you can see also that there is extensive mutation going on.

This may not be an entirely kosher comparison, but this was human PBMCs tested in vitro, where you also see a lot of mutations going on. But you do at least have some intact sequences that you see here. Again, it's a different part of the genome. I'm not trying to make a strong comparison.

If you remember, we went back sacrificed one animal, reinfected two at this time, and then followed the viral loads.

Basically we were not able to detect any plasma viral RNA at this stage. However, by proviral DNA, you get these intermittent signals that we saw for about a month. This was the same high dose of sucrose-banded virus that was given intravenously.

If you remember, at the late stage, we immunized the animals. This is one of the two that we sacrificed at nine months post-infection. Interestingly enough, in this animal we found again about 2,000 viral copies, which was completely unexpected. That suggested that the immunization and the strong adjuvant was able to actually reactivate replication-competent virus in there. I think it's fairly clear that the virus infection is established and chronic in these animals.

The next thing that we wanted to look at was what the target cells are for the virus, in the blood at least. We took the cells collected during the initial infection, days 3, 5, and 7, pooled them and sorted them in CD4/CD8 T cells, monocytes, macrophages, B cells, as well as NK cells. To make a long story short, we see that the virus is mostly in lymphocytes, T and NK cells particularly. Interestingly enough, except for the one duplicate here that was positive, we didn't see it in monocyte macrophages, which was a bit of a surprise.

In terms of phenotypic analysis, we were following these animals in the blood for different parameters, multicolor flow. As you can see, something that struck us in the beginning, as the animals -- we saw the B cells sort of spike after the initial infection, NK cells also. If you look at a proliferation marker, Ki-67, there was clearly some immune activation going on, at least in these subsets.

After the reinfection, though, although the B cells didn't move so much in terms of percent, the NK cells did, and the proliferation markers on these cells were really very marked. This is higher than what you would see in HIV infection. The T cells did show up, if you look at total T cells. When we drilled down to memory T cells, we found the same level of activation. I won't show the data.

In terms of humoral responses -- this work was done in collaboration with Abbott -- basically, we found relatively rapid humoral response to the virus. The first one was gp70, which is sort of obscured in that blot. This was redone down here with recombinant surface protein. We found responses at day 9 post-infection, followed by the transmembrane protein p15E, and then, finally, Gag responses thereafter.

Abbott has done a tremendous amount of work -- and you will hear more about it from John Hackett later on developing a high-throughput assay based on the Architect platform. This is based on microparticles bearing the different antigens that are tested both in an indirect format, where you detect the bound antibodies, or a direct format that detects both IgG and IgM.

Both formats were able to detect the response. This is just the p15E response on each of the three monkeys. The solid line is the direct one, the intermittent one is the indirect, which is shown after the initial infection.

Obviously, you can see the peak of IgM early on.

If you look at the three animals kinetically, to the different proteins that were tested -- this is the p15E you can see that after the initial infection, you get a nice response. It gets boosted in the two animals that were reinfected and then comes right back down and comes back up after the immunization. But what I would like to draw your attention to is the fact that these antibody titers come back down significantly, suggesting that, similar to other infections that don't replicate a whole lot, there doesn't seem to be a lot of antigen available to the immune system to respond.

Being pathologists, it was important to find the virus somewhere. Since we are working with monkeys, we can look at them, sacrifice them at different time points, and look for virus.

One place we focused our attention to first was lymphoid organs. We are looking at spleen, both in acute infection and chronic infection. You can see these isolated cells that were positive for XRMV gag. The same thing in the GI mucosa. This was confirmed by FISH assay against the entire genome.

The other question that we were after is, what are these cells, really? We went to looking at the jejunum as one of the examples, but it's similar in all the lymphoid organs that we looked at. We did staining for anti-CD3 T cell, XMRV.

Basically, the only cells that we see that are positive are all T cells.

The other question is -- as you all know, T cells come in many different flavors. We tested for CD4 primarily and showed again that most of the cells that did stain for XMRV were CD4 T cells.

So the virus seemed to be lymphotropic, even in lymphoid organs.
How about non-lymphoid organs? As aging males -- we have quite a few in this audience -- we're always interested in prostate.

The one thing that was really flabbergasting to us -- this is relatively low-power magnification of the prostate during the acute infection -- you can see that you have these foci of infection, which was mind-boggling, given the difficulty we had in finding the virus in the blood of these animals. There is no lack of virus elsewhere. Obviously, there was a fair amount of replication going on, or at least infection, and gag production in that organ.

At a higher magnification, you can see that all the cells we found there were these acinar epithelial cells lining the tubuli there.

In the second animal, also at the acute stage, you can see exactly the same thing. You have these acinar cells that stain.

At the high magnification, again you have these cells lining the acini of the prostate that were positive, and some of these there, which are probably just cut tangentially.

Moving on to the chronic phase, though, to our surprise, the prostate was pretty much negative by in situ histochemistry. That was unexpected.
If you look at the other male animal done at nine months, the same thing. We didn't see any signal by IHC.

However, if you drill down, not looking for protein, but looking for the viral -- really, the FISH technique that we have detects XMRV RNA -- you can see that we find cells that are still positive there. So it's not like the virus was cleared out of the prostate, but it clearly is limited. We don't seem to have any protein production. Which cells are positive -- it's very difficult to localize the type of cells by FISH.

Besides prostate, of course, a few more organs are interesting. This is the acute infection, again the lymphoid organs. We found some in the pancreas. The lung has these alveolar macrophages that are positive. In testes, we found a lot of cells, especially during the acute infection, and in the chronic infection.

Other organs, like liver and kidney, we couldn't do by IHC because of the background. So we confirmed them by FISH. There is clearly signal there. Finally, the female animals. We should be equal rights, equal opportunities. As you see, there is a lot of virus still present in the GI. But when we looked at the cervix, you had these isolated cells that came up positive, as well as in the vaginal wall.

Cervix was potentially one organ where we saw two different types of cells being infected. This is a magnification. We might find some epithelial cells, as well as some interstitial cells there being positive, which is sort of interesting.

The other one that we looked at where we saw also a lot of cells that were infected, and some of them that might be of a different lineage, is lung, where you find some that look like epithelial cells and then you have your alveolar macrophages, which are fairly positive over time.

This is just a summary table of the detection by immunohistochemistry. These are the animals at different stages post-infection that were sacrificed. You can see that in the lymphoid organs, we find virus throughout. By that time, it was very difficult to find in the blood, except for the animal that was reactivated. The GI mucosa, of course, was fairly positive. Then we had a whole series of tissues where we couldn't find it, but then the reproductive organs, as you can see, were positive, both in male and female animals, which tends to suggest maybe some hint as to how that virus may be transmitted.

In conclusion, I hope I have been able to convince you that rhesus macaques are susceptible to infection. It is a chronic, persistent infection that can be reactivated, given the proper context. But again, we don't see very much of the virus in the blood past the acute infection or some stage of reactivation.

Again, the PBMCs or the lymphoid organs that are primarily of the lymphocytic lineage are being infected. The tremendous infection in the prostate during the acute infection is something that we are following at this point also.

The other thing that we are looking at -- and we're really sort of zeroing in on some of the reasons for that -- is this transient activation of memory T cells, B cells, and NK cells, which might be another way by which -- even if the virus is not oncogenic per se, if you have constant viral replication, you may have chronic immune activation, which by itself may cause oncogenesis.

But again, in the monkeys, otherwise XMRV is clinically silent. I can vouch that they were not fatigued during the entire experiment.

So where are we going with that? One question we would like to address is, does the virus cross the mucosa to induce infection? Can it be transmitted as a model of sexual transmission? What happens in the prostate? Why is the virus controlled and not replicating constantly?

I have a "Draft" sign because this is data we are working on at this point. I got some of the data a couple of days ago. We went back, with a small grant from the Geyer Foundation, and got four macaques that we infected. We just deposited the virus into the urethra, right where you have the prostate canaliculi, and then followed the animals. Since we didn't see a whole lot, we infected back the rhesus macaques into the prostate.

I will just show you the antibody responses in these two animals. You can see that in one of the animals here, after day 50, the antibody to gp70 really shot through the roof. This was before the animal was reinfected into the prostate, suggesting that, clearly, the virus had crossed over and was able to infect the mucosa. This was the mucosa. We see that also for the other proteins.

The other thing is that there was a clear delay by the time we could find antibodies.

My last slide is just to acknowledge the people who have done the work, as well as the organizations that have funded the study. Thank you. DR.

HOLLINGER:

Thank you, Dr. Villinger.

Questions? Yes, Dr. Nelson.

DR. NELSON:

Two things. One, did you study any central nervous tissue? If there is a problem with activity, fatigue, et cetera, that would be a tissue that might be of interest.

Second, did anybody modify the activity of these animals, how long they slept or how much they ran around? You showed us tissues, but you didn't show us what they did after they got exposed.

DR. VILLINGER:

The answer to your first question is yes. I didn't put that on. You can find an occasional FISH-positive cell in the CNS of these monkeys, but no protein. In my take, this is really not a very good milieu for the virus to replicate, for whatever reason. The monkeys are fully immunocompetent.

So your second question, during the day we follow the animals. They are certainly as active as the rest of the members in that room. At night, generally monkeys are even more active, but we don't have cameras in these rooms.

DR. NELSON:

One of the last papers had how many hours the people slept when they had severe fatigue. That's one thing somebody could probably monitor. Do you know how long these animals sleep?

DR. VILLINGER:

They don't sleep more than -- well, it's hard to say. The animal handlers come in in the morning. There was no evidence that these animals were -- what you have with monkeys, especially the ones that have SIV late-stage, is that they become sluggish. You come in the room; they stop jumping around. These were no different, by any means. I really don't think that it had anything to do -- they don't lose weight or whatever.

DR. HOLLINGER:

Dr. Hanson?

DR. HANSON:

The question has been raised here about how much variation occurs in XMRV after infection. I was wondering if you have any sequence to compare to the XMRV that you used to infect these macaques. In other words, in the prostate afterwards, is the XMRV sequence a lot different than what you started with or not?

DR. VILLINGER:

That is work we still have to do. I agree. That's a very good point. We have shown that in the blood it is quite different. But again, monkey APOBEC's regime may be more active than human APOBEC's regime. That's maybe one of the limitations of our model.

DR. KLIMAS:

The degree of immune activation -- chronic fatigue patients have a very marked level of T cell and immune activation, CD4 and CD8. You said you saw more immune activation. How much more?

DR. VILLINGER:

I don't know if you saw the level. It jumps from maybe 2 percent to anywhere from 10 to 20 percent of CD4 and CD8 memory T cells. Certainly in the NK cells, which are also lymphocytes, you see marked activation. That's during the time when you do see evidence of virus in the blood. Yes, we believe there is a link there.

I think if we could induce the virus to replicate on a more chronic basis -- that's one of the next experiments that we are looking at.

DR. HOLLINGER:

Thank you, Dr. Villinger.

DR. KLIMAS:

The point is just that you have a very low viral load, and still a marked immune activation. There is more persistence --

DR. VILLINGER:

But very transient.

DR. KLIMAS:

So the activation fell.

DR. VILLINGER:

Yes. You just have this peak.

DR. HOLLINGER:

Okay, thank you.

We have three more talks in this session. We are going to do one more and then we'll take a break and do the last two afterwards and then go into the open public hearing.

The next talk is by Graham Simmons, and he is going to give us an update of Blood XMRV Working Group activities.

Agenda Item:
Update of Blood XMRV Working Group Activities

DR. SIMMONS:

The first few slides I'm going to present are very much a summary of what I presented at the last BPAC, so I'm going to try and go pretty quickly through those.

This is the panel of the research working group, including the five or six laboratories which are actively involved in the studies.

The mission of the group is really to design and coordinate research studies to evaluate whether XMRV poses a threat to blood safety, initially trying to evaluate some of the existing assays present in the participating laboratories, and then going on to establish a preliminary prevalence of XMRV in blood donors, and then hopefully determine whether XMRV is transfusion-transmitted.

We split our initial studies into four distinct phases, starting, as I said, with evaluating the performance of the existing nucleic acid test assays in the participating laboratories.

To do this, we made a number of analytical panels by serially diluting chronically infected cells or supernatant from these cells into either whole blood or plasma. These media were really chosen as they are more amenable to high-throughput screening of blood donors.

We then moved on to a Phase II study, which was pilot studies. They did give the first opportunity for multiple labs, some of whom had not detected virus before, to screen clinical samples that were found to be XMRV-positive in some labs. But the main purpose of the study was really to look at what sample type was really amenable to these assays. Most of the tests have been done on PBMCs in the published data, but we wanted to look and see whether whole blood and/or plasma were equally amenable or better for detection of XMRV, just because our repositories are based on those sorts of samples rather than PBMCs.

We also wanted to look at the timing of processing of the collected blood into these different blood components to see if that affected sensitivity of the assays.

Phase III is really an extension of that. We have a lot more positives, both from the WPI, from the Lombardi paper, and also from Harvard, from the Lo et al. studies, and also an increasing number of negatives. So we can really start to look at the clinical sensitivity and specificity of the assays involved in the studies.

Finally, our Phase IV study is blinded panels of over 300 blood donors, in order to start to get a preliminary prevalence of XMRV, both by nucleic acid and also hopefully by serological testing.

To go back to the Phase I study, as I said, it's serial dilution of chronically infected cells with a supernatant in whole blood or plasma, which were obtained from negative donors who were pedigreed by all of the participating labs, by PCR, serology, and virus culture, to be negative on at least two different occasions.

These are the results. The labs involved were the CDC, two different labs from the FDA, including the Lo lab, Gen-Probe, NCI Drug Resistance Program, and also Judy's lab at WPI. You can see from both the whole blood and the plasma panels that there really wasn't that much difference in the overall sensitivity of detection of the different assays from the different laboratories, the one exception maybe being that Gen-Probe for plasma did seem to be fairly significantly more sensitive.

assays for plasma were found to have no real substantial differences in terms of sensitivity. The overall similarity of the results suggests that the sensitivity of assays cannot explain the differences that these different participating labs found in clinical samples.

That is said with this one large caveat: 22Rv1 cells are obviously infected with a single isolate of XMRV, so it may be that this really doesn't adequately represent the diversity of all the XMRV and other MLV-related sequences that may be found in clinical isolates.

Moving on to the Phase IIa study, the WPI collected blood, using an independent phlebotomist, from four subjects who were found to be positive, either by PCR, serology, and/or culture, in the Lombardi et al. study. These were either processed immediately or put in 4 degrees for two or four days and then processed. Each sample was processed into PBMCs, whole blood, and plasma.

Unblinded panels were distributed to the WPI and CDC and a blinded panel, including some XMRV-negatives, were sent to the DRP lab at NCI for testing. The four patients were all female. They ranged in age from 25 to 50 years old. Three of them had been diagnosed with CFS over 20 years ago. One was a family member of a longtime CFS patient. Three out of four of the patients tested PCR-positive at least once for XMRV. All four were seropositive at at least one time point; several, multiple time points. And probably the gold-standard assay: All four were culture-positive on multiple occasions.
One of the patients had initiated antiretroviral drug therapy around the time that these initial samples were taken.

Looking at the CDC results, for whole blood and PBMCs, they performed nested PCR for polymerase and gag. All of these samples were negative. They also performed quantitative RT-PCR for protease, and again all of these samples were negative. Controls for beta-actin were positive in both cases.

However, when they looked at plasma, initially using a nested gag PCR assay, two out of the four subjects became positive -- interestingly, only at the day 2 and the day 4 delayed-processing samples. The day 0 immediately processed samples were negative for both of these subjects.

When they sequenced the product from these nested PCRs, it appeared to be XMRV-related.

They also performed quantitative RT-PCR for protease and for integrase. In this instance all four subjects came up positive at at least one time point -- and again, this strange phenomenon, that the day 0 was negative, but the day 2 and the day 4 were all positive. In this instance, you are unable from this product to tell the difference between XMRV and generic MLV sequence. Importantly, for all PCR-positive samples, they tested for mouse mitochondrial DNA contamination.

Similarly, the WPI results -- this is using their nested PCR described in the Lombardi et al. paper for gag, followed by sequencing of the bands for positive identification. In this instance again whole blood samples all tested negative. Unfortunately, they were unable to test the PBMCs. But on plasma, they saw very similar results to the CDC. Only one out of four of the subjects was positive at day 0, while at day 2 and day 4, all of the subjects that were tested turned out positive.

Moving on to the NCI/DRP lab results, they used a single-copy quantitative PCR assay for XMRV gag. Similarly to the CDC, they ultracentrifuged the plasma to pellet virus, whereas, in contrast, the WPI extracted directly from plasma. They also spiked an internal virus control into the plasma, a control for the pelleting step. In this instance NCI found that all of the samples by plasma Western blot and whole blood and PBMCs, and at all time points, were all negative. The plasma internal controls and the genomic DNA controls were all within range.

So two out of three labs detected XMRV in clinical samples. Plasma outperformed whole blood in both laboratories and outperformed PBMCs in the one lab that tested them, and this strange phenomenon, that day 2 and day 4 delayed-processing samples outperformed day 0 for plasma samples in both labs. We really have no good explanation for what's going on there, other than, possibly, cells may be dying and releasing either viral particles or viral nucleic acid into the plasma.

Caveats: Obviously, this panel was distributed in a mostly unblinded fashion. It's a small sample size. The third laboratory failed to detect virus, despite a phase-sensitive assay that was equally sensitive to the other two assays involved in the study. It was mainly because of this third point that we decided to repeat these studies with a more structured collection of the samples.

The samples were collected by the same independent phlebotomist at the patients' home or work. At this point, the individual who had initiated antiretroviral therapy had been on therapy for a couple of months.

We also included a pedigreed negative. This is the same negative that was used in the analytical panel, so it had come up negative in multiple assays and at least two certifications.

The blood was supplied directly to BSLI. Again, we either processed the samples the same day or, in this instance, just left them at 4 degrees for two days. We skipped the day-4 processing.

The panels were then blinded and coded and distributed to the same three labs, and also to Gen-Probe, which we included in this round of testing.
To the data. NCI performed the same assay as in the first round, except that, as well as ultracentrifugation, they also directly extracted nucleic acid from the plasma. So they did it both ways.

Unfortunately, one of the labs has yet to report the whole blood assays, so we are not presenting the data from any of the whole blood studies in this. We are just presenting plasma and PBMCs.

In this instance, NCI found again that both time points for both sample types in all of the subjects were negative. Again, the internal controls were in range.

In this round, the CDC, unlike the first round, found all the samples to be negative. Again, they ultracentrifuged prior to extraction from the plasma. They used the same assays, in addition to a couple new assays, which included a nested RT-PCR for envelope and a quantitative RT-PCR for MLV gag. However, in all of these samples, the samples were negative. Again, the controls were within range.

Gen-Probe used a high-throughput TIGRIS platform and performed target capture using a duplex assay that targets conserved sequences in two separate regions of the XMRV genome and then used transcription-mediated amplification for detection. As I showed you in the analytical panels, this is a highly sensitive assay. But again in both the plasma and the PBMCs, they were unable to detect virus in any subject at any time point.

One caveat is that it hasn't been fully validated with other MRV-like sequences. Gen-Probe is currently developing a next-generation assay that should see generic MVLs, in addition to definitely seeing XMRVs. I think Jeff is going to talk about some of this work later.

Finally, moving on to the WPI results, using the same nested RT-PCR, followed by sequencing for confirmation, they did not find the virus in any of the plasma samples. So none of the labs involved in this round of studies found virus in the plasma. They did find virus in PBMC samples in three of the four subjects. However, virus was also detected at one of the time points in the pedigreed negative. Investigation following decoding of the results determined that there had been a procedural error during the PBMC sample extraction which may have introduced contamination into this portion of the study.

So while we can say that PBMCs may have been positive, we really can't draw any conclusions from the time points that we see from these studies, as there may have been a contamination in this instance.

This is just a summary of all those results, again highlighting that three of the four labs were negative, including the CDC, in this round of testing. We also did serology assays in this round. The CDC performed Western blot for multiple MLV antigens and found all of the plasma to be negative. Frank Ruscetti at NCI performed the flow cytometry assay that Judy described from the Lombardi et al. paper and found that three of the four subjects were serologically positive, some at multiple time points. However, again, the pedigreed negative was also serologically positive at one time point.
In this round of testing, only one out of the four labs detected XMRV in clinical samples, and it was only on PBMCs. Ultracentrifugation or direct extraction of plasma did not seem to make any difference in detection by the NCI lab that performed it. A sensitive NAT assay from a diagnostic company was also unable to detect XMRV.

So based on the Phase II findings, if you look at just the Phase IIa, you could say that there was a clear advantage to delayed processing. Based on the IIb samples, we really can't say that. There doesn't really seem to be any direct, clear advantage. So rather than do this again, we are going to continue with collection of the Phase III panel and revert back to a standard next-day processing protocol -- kind of taking the middle road, if you would, and hedging our bets that way. Also I think this study highlights that we really do need to include the serology in parallel with the nucleic acid testing in the future studies.

Thank you.

DR. HOLLINGER:

Thank you, Dr. Simmons.

Questions for Dr. Simmons?

DR. BOWER:

I probably missed this. The subjects in Phase IIa and Phase IIb were the same subjects?

DR. SIMMONS:

Yes, they were the same subjects, plus the addition of an additional --

DR. BOWER:

An additional one, okay.

As far as this delayed processing, to the gentleman from the U.K. who seemed to know a lot about these viruses, have you ever experienced any advantage or do you know of any reason for delayed processing in these viruses?

DR. STOYE:

No experience. No Comment.

DR. BOWER:

Okay, thank you.

DR. HOLLINGER:

Dr. Klimas?

DR. KLIMAS:

Just on that day 4 thing. I sort of lost the reason why you went from a day 0, 2, 4, to two, to one.

DR. SIMMONS:

In the Phase IIa, there was no real difference between day 2 and day 4. If it was positive on 2, it was positive on 4. So we decided it wasn't necessary for the second round, just for practical reasons. We are really going down to day 1 because we are trying to hedge our bets -- and the fact that there didn't seem to be any difference in IIb between day 0 and day 2.

DR. KLIMAS:

It just seems like such a small sample number to make that decision, particularly when you have positive data on day 4. Why wouldn't you do day 1 and day 4 or something in Phase III?

DR. SIMMONS:

But day 1 is standard, basically. That's why we are doing that. We really don't feel that we can go around in circles doing this Phase II study for any longer. We really want to move on. And we do know from other publications that people have done standard processing and found positive samples. DR. BOWER: At least to me, Phase II did not answer the question of whether delayed processing helps. I think that's still unknown.

DR. SIMMONS:

We are initiating a separate study that is not part of the research working group that hopefully will address that.

DR. HOLLINGER:

Do you have a question?

DR. HENDRY:

Michael Hendry, CDC.

Just a couple of points on the working group. One of them is kind of to add to Jonathan's list about the biological plausibility. In cases whether prostate cancer or CFS, except for the few anecdotal reports from Lo's lab, we don't have any longitudinal data. Whether we are looking at PCR, serology, or anything else, we don't know whether these are maintained or whether they are transient responses.

From the Phase IIa study, I also wanted to point out the fact that the WPI and the Lo lab were the only two labs that reported positive signals in the negative control.

Finally, in comparing the positive serology results in the Phase IIb from WPI and the positive results from NCI, there was absolutely no concordance. That is, there were no cases where they were positive both by serology and by PCR, which is, I think, another important point.

DR. HOLLINGER:

Yes, John, last question.

DR. COFFIN:

I was going to comment a little bit further on the issue about delayed processing. I also know of no evidence in retrovirology where delayed processing, presumably leading to lysis of the cells and release of intracellular nucleic acids, which are then easier to detect, has been reported in the case of retrovirus infections. But my understanding, particularly from Dr. Busch, is that there are many other cases where that, in fact, is a very strong booster of responses.

You might want to comment on that.

DR. BUSCH:

We have seen this with PTV, sort of broad-variant category, where delayed processing results in a significant increase in plasma nucleic acids, also mitochondrial DNA sequences with delayed processing. We do have one project that is not published yet, but was presented at a meeting, where HERV-K sequences, which have been implicated in breast cancer, et cetera -- when you delay the processing, the levels of plasma nucleic acids go up, even in healthy people.

The other reason why we got into this was because, as Graham pointed out, all of the repositories that I'll talk about later that we have access to, to further study this, were processed on day 1 or 2 post-phlebotomy, and we just wanted to verify that that didn't diminish detection. We are actually interested in the preliminary data from IIa that it increased it.

DR. COFFIN:

Can I make one follow-up comment on that? In the case of XMRV, when we were trying to set up the assay for direct analysis of plasma, we tried quite hard to recover pure RNA from in vitro transcripts that was spiked into plasma. At best, we were able to recover 1 percent by sort of instantaneous processing. Free RNA does not survive in plasma more than seconds, I would say. So if there is material that's released that is detectable in a PCR assay, it has to be in some kind of a protected particle. Plasma really eats it up very fast. We rediscovered that.

DR. MIKOVITS:

I also want to correct Dr. Hendry. There was complete concordance between Frank Ruscetti's serology results and our work with those patient samples. We don't do direct PCR. We were asked to do that for the purpose of this study. We isolate virus from these people all the time. We have done longitudinal samples over decades and isolated virus from these patients and have concordance with the immune responses. So that's not correct.

DR. HOLLINGER:

Thank you, Dr. Mikovits.

It is now about 4:30. We're going to take a break until 4:45. Then we will come back and complete the last two and then go to the open session. In the open session, we will run through the talks and then ask questions later.

Thank you.

(Brief recess)

DR. HOLLINGER:

I think we'll move forward. The next talk is going to be by Michael Busch, who will discuss "Prospective and Retrospective U.S. Donor Surveillance Studies."

Agenda Item:
Prospective and Retrospective U.S. Donor Surveillance Studies

DR. BUSCH:

Thanks, Blaine. Really, carrying on after Graham's work, as he alluded to, the working group's mandate is to both execute the studies in terms of evaluating assays, hopefully identifying assays and establishing their performance characteristics, and to actually apply those tests to address the transfusion-transmission question. I want to present the design of some studies -- again, these are not yet funded, but submitted for funding -- to begin to move forward to assess the prevalence over time, as well as transfusion-transmission rates. The initial studies that I'll describe would go back to archived samples dating back almost four decades that NHLBI has put away. I'll walk through those repositories in a minute.

The concept that we have proposed is to initially test 10,000 samples, 2,000 from each of these time-defined donation repositories. For three of these repositories that are linked donor-recipient repositories, the plan would be the test a larger number of the donation specimens, if we determine prevalence is high enough, to be able to then look at the recipients and assess the rates of transmission from either seropositive or nucleic acid-positive donations to recipients, correlate transmission with viral and serologic findings in the positive donations.

These repositories are quite unique in several of the cases, in that two of them flank the period of introduction of leukoreduction. So half of these recipients received blood that had the white cells removed, which is current standard of practice in the majority of U.S. transfusions, and half did not.

Also one of these repositories, the VATS, is an HIV-infected cohort, so we could look at disease penetrance in the context of HIV preexisting infection in these recipients. There is data on mortality and morbidity in the recipients of these studies.

At least at this stage, we have commitments and have funded through the application support to Abbott and Gen-Probe to help with the testing, so we could push through the kinds of numbers we are talking about here, which would be over 20,000 donations.

This is probably a little bit hard to see. Basically, the repositories that we have selected from the larger group of what is called the BioLINCC system that NHLBI has supported over decades and now has made available through public-use access -- the repositories that we have focused on start with the transfusion-transmitted viruses study, which was collected in the late 1970s.

This is a linked donor-recipient repository, with about 6,000 donations that all went into 1,500 recipients. This is just a serum repository, so we don't have the ability to look at cell-associated virus.

We also have the transfusion safety study repository, collected in the early years of the HIV epidemic, prior to the availability of HIV antibody testing, but just before 200,000 donations were frozen down, later tested, recipients followed to look at HIV and HTLV transmission rates. The REDS repositories -- we have two of them. One of them we call the GLPR, the General Leukocyte Plasma Repository. This is just a donor repository, about 150,000 donations from 1994-1995. Here for the first time, we began to freeze down these frozen whole blood preps that were prepared one to two days post-collection by simply direct-freezing portions of the anticoagulated whole blood. So we have plasma and companion frozen whole blood.

The VATS is the viral activation by transfusion study, conducted in the mid-1990s, again, a linked donor-recipient repository. These were all HIV-infected patients who were randomized to get leukoreduced, filtered blood or non-filtered blood. They have been characterized extensively for virus prevalence and reactivation and transmission of viruses.

Finally, the RADAR repository, the most contemporary repository, collected in the early 2000s, is a donor-recipient linked repository, with plasma, as well, as frozen whole blood. There are over 100,000 donations in this repository, but 13,000 of these were transfused into 3,500 recipients where we have pre- and post-transfusion samples.

So these are the resources that we envision studying to evaluate the issues of XMRV. Again, the aim, very briefly: The idea is to test 2,000 representative donations from the five repositories that I alluded to, dating back to the 1970s. These would actually be donations that did not get transfused into the recipients. We want to first evaluate whether we have the power within the repositories to go to the most precious linked donor-recipient specimens.

The basic hypothesis -- and I do have some backup tables with the power calculations -- is that we will find prevalence in the .2 to 8 percent range and that using a combination of sensitive nucleic acid and serological assays, we will identify donors who have active viremia or evidence of past infection, and we will be able to then look at seroprevalence, viremia prevalence, over time in these donations and also examine the relationships between demographics, like age and gender, region of the country. All of that information is available.

The most critical piece, though, is the transfusion-transmission question. Our hope is that we will be able to demonstrate prevalence using established assays that we are working to validate, and then we will be able to go to the three repositories I summarized -- the TTVS, the VATS, and the RADAR -- that have linked donor-recipient specimens. Overall, there are about 11,000 donations that we propose to study that were transfused into the recipients for whom we have pre- and, in most cases, serial frequent post-transfusion samples.

The goal will be to test all the donation specimens and then, for recipients who are exposed to either antibody or nucleic acid-positive donor units, as well as matched controls, we would test the recipient post-transfusion and pre-transfusion specimens.

Again, the goals would be to correlate the transmission events, assuming that is observed, with the levels of virus in the plasma and cell compartments and the presence or absence of viral antibodies. Because in two of these studies half of the recipients were transfused with leukoreduced and half not, we can look at the effect of white-cell removal on transmission. We also have storage duration, so the time from collection of the blood to transfusion, to see if that is a variable in transmission rate.

This includes essentially healthy cardiac surgery patients in RADAR -- mostly cardiac surgery -- and then HIV-infected patients in VATS. So we can really also begin to tease out whether there is a susceptibility or disease-penetrance effect of underlying disease in the recipients.

Using a hypothesis of prevalence that ranges from .2 to 7 percent, and with 11,000 donor exposures, we should identify fairly large numbers of exposed recipients. If one assumes a 50 percent transmission rate, we'll have fairly large numbers of recipients in which to study the early dynamics of infection and look at disease outcome.

In terms of outcomes, from the aim-2 recipients, we do have clinical outcome data that was measured as part of the early studies. To the extent those data exist and the sequential bleeds that we have in the freezers are characterized, we can look at the viral loads, the sequence evolution. We can attempt to isolate virus, look at immune responses, and then correlate those with the survival and the clinical outcome data that exist in these historical repositories.

So through these studies, we believe we can contribute to the characterization of viral dynamics, immune responses, and possibly to disease associations.

Finally, a slide that we have sort of debated in the context of the industry itself: When would there be a role for a prospective screening trial? To a certain extent, you have a catch-22 here. If you haven't established that this is transmissible or is causing diseases and you move forward with prospective screening, kind of analogous to the Puerto Rico dengue issue, you pick up infected donors, but you no longer are transfusing those units, so you no longer have the ability to investigate transmission rates, et cetera. To my mind, it's premature to consider launching a prospective study.

But we have begun to think about the scope of such a study and discuss it with commercial collaborators. What we would be looking at would be at least a study of 20,000 donations. I think, given current practices, we would need to interdict the seropositive donations, so this would be a real-time study.

We could enroll the donors into follow-up and study them in terms of immunology and virology. Because this is a chronic infection, unlike dengue, these donors presumably have been infected for quite a while, so we could do what we call look-back, which is to trace prior recipients from these donors and evaluate the positivity rate in a case-control design in terms of a look-back study.

But I think the critical question that this committee is being asked to consider is, are we there yet? Is there any role at this point to undertake a prospective trial?

Thank you.

DR. HOLLINGER:

Thank you, Mike.

Dr. Nelson?

DR. NELSON:

Mike, as you know, we have a repository, the FACS study. We don't have donors, but we have a much larger population of transfused recipients and exposures. We have about 11,000 transfused cardiac surgery patients exposed to 120,000 units. We have 3,000 with essentially the same disease who weren't transfused. So there is a control.

I understand the importance of linking donors and recipients, but if the transfusion risk is lower than, let's say, one in 10,000 or something like that, this repository might be large enough to pick it up.

DR. BUSCH:

I agree with you on the FACS study that you built. I think that makes sense, probably particularly for a serologic test. You have, similarly, pre-transfusion and six-month post-transfusion bleeds?

DR. NELSON:

Yes. The economy of the testing is that you can test 10,000 or so pre and post, and get data on 120,000 exposures. In the early days, as you know, cardiac surgery patients got a lot of blood and blood units. We have also mixtures of whole blood, plasma, clotting factors. So that could all be possibly analyzed.

DR. BUSCH:

Good.

DR. HOLLINGER:

Yes?

PARTICIPANT:

Mike, I noticed that one of the repositories you were using was from the 1970s, in the initial study. Does it concern you at all that we don't know when this virus entered man?

DR. BUSCH:

That is partly what we are hopeful of contributing to. If those samples from TTVS are negative and we see accruing prevalence over time, that suggest that this is a new agent. If it has been around, if we find comparable prevalence -- 5 percent -- 40 years ago to now, and we don't have a lot of disease in transfusion recipients or transmissions, then that would reassure us that even though the virus is real and prevalent, it may not be a particularly penetrant infection.

DR. HOLLINGER:

I think Dr. Stoye said 50 million years ago.

Yes, Dr. Coffin.

DR. COFFIN:

Just a follow-up on that train a little bit, of the sequences that have been reported, their similarity to one another and to MLVs is most consistent with very short transmission chains from human to human. Assuming the virus is, in fact, getting into people, it's not impossible to consider that every little localized outbreak starts with a single mouse somewhere, because this virus clearly has very recently come out of the -- all of the sequences must have fairly recently come out of the mouse germline and might be analogous to the hantavirus outbreak, for example, where conditions, for some reason, allow this virus to replicate in some wild mouse and then spread around to humans. The same kinds of things could happen occasionally. One could have these little foci of infection of a virus that worldwide could be exactly identical from one to another, because the continuity has been carried in the mouse germline.

One has to be very careful not to -- although, as you know, I still remain quite skeptical about a lot of the issues, one has to be very careful not to think of this virus in terms of a virus like HIV. You have to sort of put what you think you know about HIV to one side, as far as things like genetic variation, epidemiology, and so on. This could be a completely different situation. We have to keep that in mind.

DR. BUSCH:

I think the one prerequisite for any of these large epidemiologic studies, though, is having throughput assays that are validated and sensitive. Right now these panels that Graham described -- we send them out, 50, 60 samples, and two or three months later we're begging for results. We need these high-throughput commercial assays in order to launch these studies.
DR. COFFIN: There's no doubt about that. But, of course, there is a lot of working back and forth. Until you have assays that you know are telling you something on samples that are validated and everybody agrees with the interpretation, putting these into enormously-high-throughput platforms does not seem exactly warranted.

DR. HOLLINGER:

Other questions?

(No response)

Okay, thank you, Mike.

The last presentation on Topic II, by Dr. Bagni, on "Assay Development Efforts on MLV-Related Human Retroviruses."

Agenda Item:
Assay Development Efforts on MLV-related Human Retroviruses

DR. BAGNI:

Today I am going to be speaking about our ongoing efforts to characterize reagents and the transition to developing a sero-assay that could be used, most likely, in research labs.

Just an outline of what I'm going to cover this evening:

- A little bit about our reagent development.
- The availability of reagents to the research community.
- Our assay development strategy.
- Some of our preliminary observations.
- Some suggestions for the path forward.

In terms of the development of reagents, working with the Protein Expression Lab in Frederick, Maryland, nine XMRV gene products from VP62 were cloned, expressed, and purified in multiple systems. The schematic shows the different protein products and their relative location on and in the virion. For the reagent development, specifically, we based the clone setup on gateway entry clones, which facilitates a lot of flexibility for researchers. We have four types of protein expression clones. We also cloned some of the gene products for protein secretion.

For the recombinant antigen production, we began with initial screening in prototype sero-assays. We have moved to small-scale production and final production on some of the antigens, now that we have believe we have an understanding of which ones might be useful in a serological assay.

These are the XMRV antigen results after purification. This is a Coomassie stain gel. What you can see are the different gene products and their relative size. All of these are running at the expected size, with the exception of TM, which in this case is expressed as a fusion protein.

To take a page from some of Ilo Singh's (phonetic) work, we attempted to verify whether the recombinant antigens that we had produced would cross-react with MLV antibodies that had been generated for MLV-related studies. Kind gifts from Sandra Ruscetti and Monica Roth to capsid RT and SU, a subset of the data that I'm showing here, show that we have cross-reactivity and would expect that these proteins could be useful in a serological assay platform.

These reagents that I'm speaking of -- specifically the clones -- are now available to researchers. They can contact the NIH AIDS Research and Reference Reagent Program. We deposited the DNAs for 64 clones. In parallel effort, we are working to generate monoclonal antibodies, which would also be available to the community -- both the antibodies and the cell lines.

We are doing this with the Antibody Characterization Lab. Some of these are actually ready to be deposited and others are still in the pipeline.

Just to speak a moment about the technical aspects of the platform that we chose to use, we decided to use the Meso Scale Discovery platform, which is an ELISA-based platform. Like an ELISA, you would coat an antigen onto the surface of the plate. If there is something to react with the antigen, such as antibodies in the serum, post-incubation, those would react with the antigen. You would come in with a labeled secondary. This is labeled with a proprietary molecule from MSD. With the addition of electricity and co-reactants from their proprietary buffer, you will have the emission of light. In the absence of electrical current, your background is really zero. Each of these individual spots can be quantified using software, and the light emitted is captured by a CCD camera.

Here is a discussion of the platform and the reagents that we have developed. This slide is really the transition: How do we go from having reagents which we believe would be useful in a sero-assay to actually having a developed sero-assay that would be useful to the research community? There are several hurdles, I suppose, that need to be addressed and overcome. The top three, in my mind, are listed on this slide.

To begin with, we don't know what the prevalence of the virus is in the general population. Depending on the strategy that you use to develop these assays, positive subjects in the normal donor population could confound your results and/or you may not be able to discriminate between cross-reactivity or lack of specificity in your assay, if that were to exist.

We also are still trying to characterize positive and negative samples. Not to make light of the situation, but in the case of the sero-assays that we are looking to develop, we have the situation of a little bit of a chicken and egg. We need a clinical control to validate the assay and we need an assay to validate the clinical control. That is definitely something that, if it were addressed, would make things a little bit easier.

Finally, we do not know the levels of antibodies in XMRV-positive subjects. We don't know when subjects are being infected. We don't know what the immune profile is. This makes it difficult, when data is discordant, to know whether it is the assay or whether it is something to do with the actual pathogenesis of this virus.

Some of the limitations, which I have touched on in the previous slide, but to emphasize moving forward in this talk:

The preliminary assay characteristics for the assay that I'll be describing are calculated from a very small sample number. We obviously are continually working to increase this number, but we are still working with a fairly small n.

We are also making an assumption of serostatus. We are assuming that most donors are negative, and if a subject has been classified as XMRV-positive by another test, they will be serologically positive as well.

Again, the lack of immune profile information is confounding.

I'm not the first to say this this afternoon, but if there were bona fide, pedigreed, in this case not nucleic acid, but antibody clinical controls, this would certainly would help the field move forward.

To show that, besides on a Western blot, we could actually use our platform and detect monoclonal antibody reactivity to our antigen, we diluted an SFFV Env monoclonal antibody manyfold. Knowing the initial concentration of this monoclonal antibody, we are in the low nanogram-picogram range of detection using our platform and this monoclonal antibody.

We also titrated a polyclonal capsid. Although it appears to be slightly less sensitive, based on some of our results, we assume that this still has some utility in a serological assay.

The qualification of XMRV recombinant antigens -- the strategy that we chose was that we obtained 77 donors from the NCI Frederick Research-Donor Program, as well as donor plasma that had been archived in our freezer since the early 1990s. Those were obtained from BBI Diagnostics. We also included 39 XMRV-positive subjects from the initial Lombardi paper that were CFS patients. Without bias to which proteins might be antigenic in subjects, we assayed all nine gene products, recombinant products, to this training set. Then we used statistical analyses for each to determine if any of them had utility in a screening assay.

The statistics that we used were the receiver operator characteristic curves. There are a few parameters that I just need to touch on so you can appreciate where we are with the data at this point. Sensitivity is the proportion of patients with the virus that will be reactive on the test, and specificity is the proportion of the subjects without the virus that will be non-reactive on the test. What you want to see when you plot these two values of sensitivity and 100 minus the specificity is, you would like to see your curve approaching the upper left-hand corner. This would be a true reactive rate, 100 percent sensitive and relatively 100 percent specific.

If we look at the ROC curves for integrase and reverse transcriptase, we can see that this is not approaching the upper left-hand corner at all. In fact, it is along the diagonal, which suggests, actually, that if you flipped a coin, you would have a better chance of predicting this.

However, if we move to the ROC curves for capsid, TM, and the surface unit, SU, you can see that we're doing a little bit better. In the case of SU, this data is encouraging. I need to caution that this data set we have been able to replicate with one lot of SUU protein that we produced; however, subsequent lots have not performed as well.

We have identified the production issue, and we are going back and addressing that, moving forward.

If we look at the training set, understanding that this data is taken directly from the training set -- so it's not blinded and we are just classifying how well we did -- for the subjects, of which we had 39, we see that 10 of these actually have reactivity above background for capsid, transmembrane, and surface unit. Then if we look at the donors, there is actually one out of the 77.

Something that we still need to work on is, what is this background where we see that some of the antigens actually have reactivity that isn't concordant with the other antigens? This is certainly something that we need to follow up on, to understand what that really does mean.

I would like to take a minute, with the caveat that these are preliminary findings and raw, non-calibrated data, meaning that we do not have a calibrated control -- when we looked at 1,000 donor samples that were provided to us by Dr. Harvey Alter -- 500 of these are NIH donors and 500 come from the Children's National Medical Center -- and we assayed for capsid, TM, and SU -- the SU, unfortunately, I'm not able to report at this time -- we see that the concordance between the two assays is around 5.5 percent. Again, we have this background, which we definitely need to address, in addition to, which proportion of this 5.5 percent are false positives or in the reminder, what might be actually false negatives. Until we actually have a better understanding of the prevalence in the human population, these are harder numbers to get to.

In summary, multiple XMRV recombinant antigens produced by the Protein Expression Lab have been used in sero-assay development. We are able to detect reactivity to capsid, TM, and/or SU. I did not show this data. Some subjects, as well as donors, are reactive to p12, matrix, and nucleocapsid, but in the absence of clinical controls, the statistics are not conclusive. We suggest that we can include antigens that are reactive in human sera into a positivity algorithm and suggest that the requirement to be positive to two out of three or three out of three might lend some insight into what the serostatus for XMRV subjects might be. Our ongoing efforts:

We are continuing development of this. It's a daily activity in the laboratory. Of course, we would like to refine our cut-points. I think clinical controls will help with that.

We will work to see if we can come up with a positivity algorithm or a reactivity algorithm.

Secondary assays, such as Western blots, nucleic acid tests, some of the tests that John Coffin has mentioned, in addition to single-genome sequencing. These are all complementary, and until we understand what is necessary or not necessary to say that a subject is XMRV-positive or antibody-positive, we need to consider that all these are definitely part of the game still.

Not to belabor the point, but pedigreed clinical controls will help with this.

Samples from experimentally infected animal models are an option. However, they aren't a substitute for clinical controls.

But in the absence of not coming across this much more quickly than we probably expected to, the NCI has moved to generating animal models. Jeff Lifson, who leads the AIDS and cancer survivors program at NCI Frederick, has purified XMRV virions. This is an HLPC fractionation. You can see that's an incredibly pure prep. This is SDS-PAGE and immunological analysis using other MLV antibodies. We can see cross-reactivity to the native purified XMRV virions.

To finalize, I'll say that this is where we are today. Our preliminary assay characteristics, yes, were calculated from a small sample number, but it gives us an idea of where we need to be going. We look forward to analytical performance panels and clinical controls to help with this. In the meantime, we will obviously be using the macaque infected models that the NCI is currently working on. But we will still hold out that this will be the ultimate control for the assay.

I need to acknowledge the people in the Protein Expression Lab, led by James Hartley, who cloned, expressed, and purified all the proteins, the Molecular Detection Group, which is my laboratory, which has been working on the development of the assay, Jeff Lifson, who generated and purified the virus, and Denise Whitby and Nazarena Labo, who helped with the statistics. Funding was from the CCF office of Bob Wiltrout and Stuart Le Grice. And thank you to Frank Ruscetti and Kathy Jones for support. Harvey Alter provided the 1,000 donor samples, as well as Judy Mikovits for the WPI XMRV-positive subjects.

Thank you.

DR. HOLLINGER:

Thank you.

On the samples that you had -- maybe I missed this -- on the samples that were antibody-positive for the capsid, transmembrane, surface, et cetera, were these also nucleic acid-positive? Which ones, as it relates to the antibodies?

DR. BAGNI:

Yes. If they came from the WPI cohort, then they had tested nucleic acid-positive in their hands. We ourselves did not do any subsequent testing. We have not followed up on any of the donor results from the 1,000 donor samples.

DR. HOLLINGER:

So all the ones that you got from WPI were positive.

DR. BAGNI:

Yes.

DR. HOLLINGER:

Was there a distinction, then, in what kind of antibody you found in those?

DR. BAGNI:

Not that I am aware of, no.

DR. HOLLINGER:

Okay. You might think that maybe if it's surface antibody, it might be a lot different than it would if it's one of the other antibodies.

DR. BAGNI:

We know there are 20-some subjects that were surface unit-positive. Only 10 of those were positive also for capsid and TM.

DR. HOLLINGER:

Thank you.

Dr. Klimas?

DR. KLIMAS:

So these were serum versus plasma serum?

DR. BAGNI:

These were actually mixed. We mostly are using serum, but we had some plasma in there.

DR. KLIMAS:

You sort of morphed from XMRV to MLV in your language. I wasn't sure if it was across all cases.

DR. BAGNI:

These are antigens that were cloned from VP62, which is XMRV. However, we used reagents, because that's all we have access to, that are MLV reagents the antibodies.

DR. KLIMAS:

And they worked?

DR. BAGNI:

And they worked reasonably well.
DR. HOLLINGER:

What is "reasonably well"?
DR. KLIMAS:

.62, .72, and .86. Those are good ROC curves. I like that.

DR. BAGNI:

The ROC curves are subjects. I do not know -- they are from the WPI study, so that is XMRV.

DR. KLIMAS:

The first two are lousy, but --

DR. BAGNI:

But those aren't included in the analysis. That was just to show that not every XMRV antigen performs equally.

DR. KLIMAS:

But PSA has a ROC curve of .62, and we use it every day. And that was your worst of those last three.

DR. BAGNI:

Sure. So we could say that we would see cross-reactivity to MVLs. But because it's a serological assay, there is always the question of specificity, in general.

DR. HOLLINGER:

Any questions from the committee?

(No response)

Thank you very much.

We are going to move on to the open public hearing. We have told the speakers how much time they have. We'll see whether we are going to ask questions of them right after their talk or go to the end.

Agenda Item:
Open Public Hearing

Again, I must read the announcement for the open public hearing, so bear with me. Both the Food and Drug Administration and the public believe in a transparent process for information gathering and decision making. To ensure such transparency at the open public hearing session of the advisory committee meeting, FDA believes that it is important to understand the context of an individual's presentation. For this reason, FDA encourages you, the open public hearing speaker, at the beginning of your written or oral statement, to advise the committee of any financial relationship that you may have with any company or any group that is likely to be impacted by the topic of this meeting. For example, the financial information may include the company's or a group's payment of your travel, lodging, or other expenses in connection with your attendance at the meeting. Likewise, FDA encourages you, at the beginning of your statement, to advise the committee if you do not have any such financial relationships.

If you choose not to address this issue of financial relationships at the beginning of your statement, it will not preclude you from speaking.

With that in mind, Dr. Harvey Klein will be giving a statement from the AABB. Dr. Klein?

DR. KLEIN:

Thank you, Mr. Chairman. My name is Harvey Klein. I'm the chairman of the AABB's Interorganizational Task Force on XMRV. I have no conflicts of interest.

AABB is an international, not-for-profit association representing individuals and institutions involved in the field of transfusion medicine and cellular therapies. AABB membership consists of nearly 2,000 institutions and 8,000 individuals. Members are located in more than 80 countries.

AABB thanks you for the opportunity to participate in these discussions today by offering our perspective on the questions that have been posed to the committee. For more than a year, AABB has been analyzing the findings of various research groups studying XMRV/MLV as they seek to understand whether infection by these agents causes human disease. In December 2009, AABB formed an interorganizational task force that includes representatives from the blood community, a patient advocacy representative, XMRV subject-matter experts, and liaisons from a number of government agencies. The task force was charged to review available data, make recommendations for action to assess and, if necessary, mitigate risk, and develop appropriate educational materials for donors, medical personnel, and the public on the risk of XMRV transmission through blood. AABB has assembled a second task force that is focused on these same issues as they relate to cellular therapies.

As has been already noted here today, AABB issued an association bulletin, 10-03, in June of 2010 recommending that blood collectors, through the use of donor information materials available at the donation site, actively discourage potential donors who have been diagnosed by a physician with chronic fatigue syndrome. I'll refer to that as CFS, since it has many other designations. Educational materials were provided with the association bulletin. This proactive step was taken on the advice of the task force as an interim measure following the article in Science in October of 2009 that reported the association between XMRV infection and CFS. At the time the association bulletin was published, the task force was also aware of the Lo study which was subsequently published in the Proceedings of the National Academy of Sciences in September of 2010, and considered these findings in making its recommendations.

A policy of the active provision of educational materials to discourage potential blood donors from donating when they have particular illnesses or symptoms is a tool previously approved by the FDA which has been used successfully for many years by blood collectors. AABB believes this interim measure is appropriate and sufficient, based on the available scientific evidence. The recommended process is, to our understanding, the most active approach to deferral of potential donors with CFS being used by major blood services around the world and preferable to introducing unvalidated screening questions.

Implied in the AABB recommendation is that potential donors who identify themselves during the donor interview as having received a diagnosis of CFS from a physician would be indefinitely deferred. Information about the number, the rate, and the demographics of these donors who self-identify is accumulating and at this point in time the numbers are not large.

AABB standards already require that prospective donors be in good health and be free of cancer. In general, donors who present with a history of prostate cancer are deferred unless they are disease-free and no longer receiving therapy. These donor eligibility criteria for prostate cancer are supported by several epidemiologic studies that have shown no association between prostate cancer and a history of blood transfusion. Therefore, the association bulletin recommendations did not include a change in the deferral criteria for potential blood donors with a history of prostate cancer.

The Blood Products Advisory Committee has been asked to consider the issue of donor testing for MLV-related retroviruses even in the absence of confirmed disease causation. Absent evidence that these viruses have a causal role in any human disease, it seems reasonable that the following criteria be met prior to the implementation of donor screening:
Evidence of transfusion transmission of these viruses.

Consistent evidence of association of these viruses with disease.
Development of validated assays for these agents that detect infected individuals but do not implicate non-infected individuals.

Since no causal association of XMRV with human disease has been demonstrated, a decision to introduce a blood donor screening assay, were one to become available, would appear to be premature. Many commensal viruses -- for example, Anellovirus -- are known to be transmitted by transfusion, but despite extensive study have not been associated with disease. In the absence of direct evidence of causation, a decision to implement testing should be based on the assessment of recipient risk that includes the prevalence of the infection in the donor population, the transmission rate to recipients, and the current best assessment of the risk of recipient harm, compared to currently accepted risks of transfusion.

Members of the blood community are concerned about the potential threat to the blood supply posed by XMRV/MLV and are actively involved in efforts to validate quality-control panels and develop tests for the detection of MLV-related retroviruses. However, we believe that current evidence does not support introducing any test methodology at this time. Furthermore, there are as yet no firm data to compare the efficacy of NAT versus antibody testing methods. AABB expresses its gratitude to the patients with CFS who are participating in these research projects and providing invaluable specimens for conducting the studies.

AABB remains committed to monitoring activity and supporting research associated with XMRV, taking steps as appropriate to ensure the safety of transfusion recipients and of patients receiving cellular therapies, and participating in a dialogue with the FDA as necessary to further this goal.

Thank you.

DR. HOLLINGER:

Thank you.
Any questions for Dr. Klein? Dr. Demetriades?

DR. DEMETRIADES:

Dr. Klein, do you perhaps have any statistics about the number of patients with chronic fatigue syndrome who want to donate blood?

DR. KLEIN:

We have some data -- and I think there are others in the room who have gathered this -- on the number of individuals who have self-deferred since the AABB policy was introduced.

Perhaps Dr. Stramer, if she is still here, wants to talk about the Red Cross data. I think perhaps Dr. Bianco may have some other data.

DR. HOLLINGER:

Sue, I think you data from the others, so maybe you could just pool them together or at least mention them.

DR. STRAMER:

Let me first talk about the Red Cross data. We implemented a procedure, based on the educational materials released from the AABB, on August 31. It was fully implemented in our regions by October 11. Over the next two months, we have queried our database to find out how many donors have stated at preregistration or post-registration that they have had a medical diagnosis of CFS.

In the two months that we have been doing this, or for over 1 million donations, which is about 1 million donors, we have now had 34 individuals who self-deferred. That's 34 in 1 million, or .003 percent. So it's about 1 percent of the general population that we believe may have CFS, assuming .3 percent of the general population does have CFS. Just like other deferrals we see, based on, "Are you feeling healthy and well at the time of donation," donors who present are a preselected population and would be expected to have lower rates than observed in the general population.

Of the 34 that I mentioned, the mean age is 52, 25 are females, 30 are Caucasian, and nationwide they represent a mix from widespread geographic areas.

Regarding the ABC data, Lou, why don't you just mention your experience?

DR. KATZ:

Implementation among the independents is proceeding apace. I think we are at about 75 percent now. I expect over the next several weeks that we will begin to approach 100 percent.

At my center, which is slightly smaller than the Red Cross system, where we started on August 2, we have had 10 donors self-defer out of 50,000, and 75, the last time that I queried our computers -- so the point estimate rate, a hair higher than the Red Cross, but the confidence intervals, completely overlapping.

We are using a little bit different educational materials than the Red Cross. We are asking people who self-defer to provide us with contact information so we can do a more complete interview regarding where their diagnosis was made. We're doing that on the fly.

The demographics of our 10 look just like the demographics of her 30-something.

DR. KLEIN:

I can also add to that Canadian Blood Services, which I don't think is represented here today, instituted the same self-deferral policy at about the same time. Their numbers are also comparable -- small numbers, but definite deferrals.

DR. RENTAS:

Dr. Klein, this morning we heard a joint statement from the AABB, ABC, NARC. This afternoon we did not. It was just pretty much an AABB statement. Is it safe to assume that not everyone that is part of this organizational task force agrees with your position?

DR. KLEIN:

It was the agreement of the task force. But as you know, with so many different members representing different organizations, we don't have an official position of every organization represented on the task force -- the government agencies, the Canadian agencies, the non-government agencies. It is the position that was recommended by the task force and adopted by the AABB. This is the AABB's position.

DR. DEMETRIADES:

Since the number of self-deferred donors is so small, why don't you use a stronger term -- instead of saying "actively discourage," exclude them?

DR. KLEIN:

I think with the currently available data, this is a prudent and sufficient approach. I think that's what the task force agreed with as well. I'm not sure that stronger terms, certainly in our statement, are necessary. Certainly we recommend strongly that people who have a medical diagnosis self-defer. I'm not sure there's much more strongly that I can say.

DR. STRAMER:

The procedure that the donors see say, if you have a medical diagnosis of CFS, please let us know. Then they are indefinitely deferred. It's just a little bit of semantics when you talk about what is in the AABB's association bulletin versus translating that into operational procedures.

In the actual blood center operations, the donors are presented clearly with information that says, if you have had a medical diagnosis of CFS -- it's a full-page information sheet -- please let us know, and you will not be allowed to donate today, or until further information is available.

DR. COFFIN:

Do you have any information or any way to find out how many patients who have been diagnosed with CFS fail to self-defer?

DR. KLEIN:

How many subjects who have a medical diagnosis fail to self-defer? No, at this point there's no way of knowing that.

DR. HOLLINGER:

I think it is important, just for others in the audience who may not know, that there is a difference -- indefinite deferral is not permanent deferral. I think that's always important, because some people feel that when you say indefinite deferral, you never could donate again. It's a sort of interim type of deferral.

DR. KLEIN:

Thank you. It is both an interim measure and an indefinite deferral until more data are available.

DR. HOLLINGER:

Any other questions of Dr. Klein?

(No response)

I think we'll go on. Another person who has asked to speak today is Kimberly McCleary.

MS. MCCLEARLY:

This actually follows nicely the previous discussion.

My name is Kim McCleary. I am president and CEO of the CFIDS Association of America. The CFIDS Association of America is the largest and most active organization working to make CFS widely understood, diagnosable, curable and preventable. For 23 years the association has supported research through more than $5 million in direct grants, sponsored scientific symposia and research think-tanks, sought to effect more responsive public policy and has widely informed the patient community, the media, the medical community, and researchers about the severity of CFS and the individual and collective toll it exacts.

I appreciate the opportunity to address BPAC on the topic of blood safety. Earlier this year -- and I'll note that it was prior to implementation of the AABB guidelines for CFS -- our organization used a Web-based survey tool to administer a 50-item questionnaire about possible risk factors for CFS.

Questions were designed to analyze information based on population norms from larger national surveys like NHANES. Four items related to blood donation and transfusion experience.

Those four items are:

- Have you ever received a blood transfusion
- Have you ever donated blood or blood products?
- How many times have you donated blood or blood products in the past 10 years?
- How many times have you donated blood or blood products since being diagnosed with CFS?

One thousand seven hundred forty-seven individuals responded to the survey. Ninety percent of respondents had been diagnosed with CFS by a physician. Eighty-six percent were women, and the average age was 57 years. Of the 1,529 people who answered the question about blood transfusion, 124, or 8 percent, indicated that they had a blood transfusion prior to becoming ill with CFS and 50, or 3 percent, reported that they received a transfusion after being diagnosed with CFS. The figure for blood transfusion prior to CFS, the 8 percent, is not significantly different from the general population as a whole, especially when adjusted for age and sex. However, retrospective donor-linked studies should investigate this issue with more rigor.

One thousand five hundred thirty-one people answered the question about having ever donated blood. Forty-two percent reported ever having donated blood. Those who reported no history of blood donation were not asked to respond to additional questions about the timing or frequency of blood donations.

Thirty of 650 respondents indicated that they had donated blood in the past 12 months and 225 responded that they had donated blood one or more times over the past 10 years. Of perhaps greatest interest to this committee -- and somewhat a response to Dr. Coffin's question -- is that 115 of 640 people who answered the question indicated that they had donated blood one or more times since being diagnosed with CFS.

There are obvious limitations to Web-based surveys, but these results reinforce the need to expand efforts to educate potential donors about CFS. We commend the American Red Cross and independent centers that have already taken this step and now indefinitely defer individuals who indicate a past or present CFS diagnosis.

Many in the CFS community anticipated that the studies being led by the Blood XMRV Scientific Working Group by now would have yielded more definitive information about risks posed to the blood supply by MLVs and the feasibility of wide-scale testing of blood donations. Today's presentations indicate that these issues cannot yet be resolved.

However, the lack of conclusive data does not impede the FDA's opportunity to take action that will further safeguard health without an injurious impact on the availability of blood for those who need it.

According to the FDA website, a person's suitability to donate blood depends on two general considerations: that the donation will not be injurious to the donor, and that the donated blood will not be unnecessarily hazardous to the recipient.

It has long been the association's guidance to CFS patients that they not donate blood or organs out of concern for the safety of both the donor and the recipient. Research has demonstrated that orthostatic intolerance, low blood volume, and infections with a variety of agents are common in CFS. While more information may be needed to assess the potential threat posed by MLVs and the prevalence of this family of retroviruses in CFS patients, there already exists sufficient evidence in the 5,000 peer-reviewed articles about CFS to support an FDA policy of indefinite deferral of individuals diagnosed with CFS. This policy would be consistent with the practice being followed by most blood collection centers in the U.S. now. Based on preliminary data on deferral rates, such a policy is reasonably achievable without unduly decreasing the availability of this lifesaving resource, a policy requirement stated on the FDA website.

The CFIDS Association of America urges the BPAC to respond affirmatively to the FDA's first question about CFS, and we restate our strong support of a policy to indefinitely defer individuals diagnosed with CFS.

DR. HOLLINGER:

Thank you. Any questions?

DR. NELSON:

Could we get a copy? Are there copies outside? That was an excellent presentation. I would like to have a copy of it.

MS. MCCLEARY:

I can email it. I had copies to bring with me, and I left them behind, unfortunately. But I can make that available to the committee.

DR. NELSON:

These are the best data I've heard so far on this.

DR. HOLLINGER:

It's good, but let's remember that this is a Web survey. If you look at the Web survey, I think one of the things, for example, says that 90 percent of the respondents had college or higher education, which I think is probably a lot higher than you would find in the CFS population at large.

Am I correct in that?

MS. MCCLEARY:

Actually, among diagnosed patients, probably not. Dr. Klimas can respond to that.

DR. KLIMAS:

A very important point. Less than 84 percent of patients with this illness are undiagnosed. Only 16 percent are aware of their diagnosis. So it's a very big concern. And, of course, they are the ones that can really push through barriers of health care to get to their diagnosis.

DR. NELSON:

Despite that, these are interesting data.

DR. HOLLINGER:

It is. But I think you have to take this in the context of the type of survey.

DR. NELSON:

You have to take all data in the context of --

DR. HOLLINGER:

Can I quote you on that?
Any other questions for Kim. Dr. Key?

DR. KEY:

I'm interested in the position of your organization and perhaps a core group of individuals. Is it pretty uniform that a person who is deferred with this diagnosis is surprised when they are? What is the reaction? Is it homogenous or is it polarized in terms of being deferred? Do you hear feedback on that?

MS. MCCLEARY:

When we remind our readers and constituents of the policy, some people are surprised that they hadn't heard about it before, because their physician or they have heard otherwise that there's no reason they shouldn't give blood.

Of course, this would all predate the beginnings of donor education activities. For most people, they say, "I'm in no shape to give blood anyway. Why would I do that? I'm dizzy and feel awful most of the time. Of course I wouldn't give blood. Why would you even have to remind us of that?"

So there are some mixed reactions to that. Nancy can probably speak to that in her practice. The remitting/relapsing nature of the condition -- and I think maybe the data that Dr. Hanson shared about the periods of fewer symptoms and relative wellness compared to the more severe periods -- do make this perhaps more of an issue for people who don't have a severe case and are bedbound and homebound. There are people that are fairly functional who also carry this diagnosis. Maybe Nancy wants to say more about that.

DR. KLIMAS: Just that I think you will find in most cases that the patients and the advocacy community find that the risk of this infection -- actually, it's the first time they are being taken very seriously in 25 years. So it's almost a validation. That's why you might see some of the language in the open testimony things here that is so passionate. It's because they have been soundly dismissed. When I say that only 16 percent of the cases have been diagnosed -- and that's based on some very important epidemiologic work that has been done -- that describes how poorly we have been educating physicians and how terribly these patients -- if you could hear the stories, it's just pitiful, the way these patients have been treated.

This infection, the other infections they have -- there is a lot of objective data on how ill these patients are. But for the first time, it feels to them like there is a governmental response saying, "Oh, my God, you're really sick." I think that's what you are hearing here.

DR. HOLLINGER:

Just to clarify, Dr. Klimas, there are 600,000 patients diagnosed with CFS in this country, confirmed?

DR. KLIMAS:

They are not diagnosed. There are roughly a million-ish, between 800,000 -- there is this wide range, depending on the case definition, but say a million. In that, there are about 16 percent that are diagnosed. So that's 160,000.

When they self-defer, how are they going to do that. If 84 percent don't know they have this illness, you would almost have to ask them if they -- you almost need the little checklist: Do you have five of eight symptoms? Have you been profoundly fatigued.

DR. NELSON:

Would you suggest that the donor exclusion criteria use the diagnostic criteria, then?

DR. KLIMAS:

It could well be. It's a wonderful opportunity, actually, to educate the public about the illness, because when they go in to donate blood, they might learn a little something. A little handout that says, "This is what the illness is. Do you think you might have it? If so, self-defer," might be an easier way than to say, "Have you been diagnosed or haven't you?"

DR. HOLLINGER:

Dr. Demetriades.

DR. DEMETRIADES:

At least theoretically, patients with CFS should feel significantly worse after donating blood. Do you have any information on this issue in the population.

DR. KLIMAS:

Oh, yes, they do.

MS. MCCLEARY:

The orthostatic intolerance and problems with autonomic nervous system function or dysfunction are profound and have been documented by a number of centers, beginning with Johns Hopkins and the University of Miami, and David Streeten's work also showing lower-than-normal blood volume. They do have relapses, even after giving blood for normal laboratory studies or if they are involved in a research study and they have to give many tubes to participate in a certain study.

DR. DEMETRIADES:

Are these published studies?

MS. MCCLEARY:

Yes.

DR. NELSON:

Should blood banks then not transfuse blood from a donor that collapses after donating? That might be a diagnostic criterion.

DR. HOLLINGER:

Ms. Baker, do you have a question?

MS. BAKER:

Yes. I was wondering to what extent you have been working with any of the prostate cancer advocacy groups on this issue.

MS. MCCLEARY:

We have not found a lot of interest among the prostate cancer groups on this topic, believe it or not. I have wondered about that myself, why there is not greater engagement from that community in meetings like this one.

DR. BOWER:

I just wanted to follow up on what Dr. Klimas said. If only 16 percent of people know they have been diagnosed, then actually both the educational material and this proposed question, "Have you ever been diagnosed with chronic fatigue syndrome," probably wouldn't screen out the majority anyway. "Do you feel well today?" may actually be the best screening question. Just a thought.

DR. KLIMAS:

Think of it sort of MS-like. There is sort of a remit/relapse course. There are periods of wellness, sometimes even years of wellness. So I don't know. I don't think that would totally solve the problem. It would help a lot, but it wouldn't necessarily solve the problem.

DR. GLYNN:

I just wanted to ask for a little bit more clarification on what exactly is on the donor education materials. Do you just have the question, "Do you have chronic fatigue syndrome?" Or is it more detailed, giving a little bit about the symptoms?

DR. STRAMER:

I don't have the AABB association bulletin with me. It has two attachments. One is a placard that is very general information about chronic fatigue syndrome. That is what we include in every donor's pre-read materials. They see a picture of an individual. It is just very preliminary information -- if you have a medical diagnosis of CFS, please let us know. It's very generic. Then, for any donor that does let us know, we provide them an information sheet.

That was the second attachment in the association bulletin. The information sheet provides links to the CFIDS website and to NCI, so that donors can get more information, rather than a general information sheet that the donors may not read. We provide basic information and then the links where the donors can get much more specific information.

DR. KLEIN:

If I could just add to that, there is no question. The question is, "Do you feel well today?" The informational materials provided are as described by Dr. Stramer. But they are examples. Many blood centers have elected to give more information. Some have decided to give less information, with the feeling that you can overwhelm people with details. That's really up to them to decide.

DR. HOLLINGER:

Go ahead.

PARTICIPANT:

Excuse me, I know I am not signed up to speak, but may I have 60 seconds, as a CFS patient?

DR. HOLLINGER:

(Off-mic)

If there are no other questions, I think we'll move on to the third person who has asked to speak. That will be Dr. Jeff Linnen, from Gen-Probe.

DR. LINNEN:

I just want to give a brief update. I'm from Gen-Probe, in San Diego. We're a molecular diagnostic company. We are in a partnership with Novartis for blood screening. A large proportion of the blood in the U.S. that is NAT-screened is screened with our assays. So we really feel an obligation to be involved, in whatever way we can, in terms of research to try to understand what the threat is to the blood supply for XMRV or MLV-related viruses.

These are the objectives of the XMRV work at Gen-Probe. The first objective was to develop a prototype assay that would work on our high-throughput system. We really wanted to make the assay as sensitive as possible.

I'm going to actually talk about two versions of an assay. For the first assay we developed, we're not really sure how broad the specificity of this assay for detection of related viruses. With the second one, we have really opened up the detection capabilities for all murine leukemia viruses. We want to look at a lot of different specimen types, including urine, which is one thing that hasn't been mentioned here. We do have prostate tests that work in urine. We also, obviously, want to get our hands on many chronic fatigue syndrome samples. We have a number of studies planned for that. Then, in collaboration with Mike Busch and Sue Stramer, we want to look at the possibility of transfusion transmission.

Another interest to Gen-Probe is to look into detection in prostate cancer samples a possible way to diagnose prostate cancer. This is the high-throughput system that you have heard something about.

But before I talk about this, just a few reminders about the assay. This is a TMA assay. The amplification -- this is not PCR. It's based on transcription-mediated amplification. The assay that was used in the studies that were discussed earlier is actually a duplex assay that targets two different regions of the XMRV genome. We tried to make some guesses about what would be conserved, because there weren't a lot of sequences to work from when we initially got involved in the work.

It runs on the TIGRIS system. By high-throughput, we mean about 1,000 results in 14 hours. The time to first result is about three and a half hours, and you see 100 results about every hour. The great thing about the system is that it has process controls for all assay steps. All of our assays have internal controls to validate each individual reaction.

Here's a little bit of the analytical sensitivity data. This is really consistent with the data that Graham Simmons showed. It's actually two different experiments. On the left we are looking at transcript that's synthesized in vitro, and on the right we are looking at a virus. If you look at the table at the bottom, those are the results from doing what's called a probit analysis. It's a type of regression analysis from a dilution series. You can calculate at what copy level the assay would detect 95 percent of the time. With the transcript, it's around 17 copies. With the virus, based on an estimated value, it was about 2.5 copies of the virus.

I would just conclude from this that we think the assay is pretty sensitive. The first screening that we did after we put together the assay was to look at a collection of normal donors that we obtained from Susan Stramer at the American Red Cross. These were all from the Charlotte, North Carolina testing site. We screened them on the TIGRIS system. We looked at a total of 1,435. The results were all non-reactive. One of the conclusions we make from this is that the assay is not prone to false positives. We try to get as much as we can out of all the data.

We also had some samples from a previous study, where we were looking at HIV in plasma versus whole blood. We had remainders of the whole blood samples. We had a total of 44 HIV-infected blood donors. These were identified in blood screening. These were samples that we received from Mike Busch. I'm presenting the data a little bit differently here, but the same result: Out of the 44 whole blood samples that were tested in our assay, we saw no samples that were reactive for XMRV.

Based on those negative results, we decided that it would probably be useful to make some modifications to the early prototype assay. So, as I mentioned in the beginning of my talk, we have modified the assay, where it will detect a very wide range of murine leukemia virus-like sequences.

The testing scheme may end up being a little bit more complicated, because we're not quite sure what the results will mean if they are positive. It may require a lot more additional testing to understand exactly what we are detecting.

In addition to broadening the range of detection, we have also made some changes to optimize the assay. There are some chemistry modifications that we can make to the assay to increase the sensitivity for DNA. This assay, we think, will have equivalent sensitivity for both RNA and DNA.

What I'm showing here is some preliminary analytical sensitivity data, similar to what I showed for the earlier version of the assay. What you can see in this experiment is that the analytical sensitivity is very similar. The 95 percent detection level is estimated to be I can't read it, but it looks like 16.5 copies. So we think we're ready to go with this assay. We want to do a similar kind of study with maybe about 2,000 normal donors -- we are in the process of getting those samples right now -- to get an idea of, with an assay design like this, what you detect in a normal blood donor population. We should probably be doing that in the next week or so.

What do we have ongoing right now and planned for the future? You have heard that we are working actively with the NHLBI-sponsored working group. Some of the data was presented earlier today. We are very grateful to be included in those studies. We would like to take part in the additional phases that are planned.

As I mentioned before, we are working on arrangements to get as many CFS patient samples as possible. Really, the way I'm looking at this, I would just like to screen as many as we can. We can easily, in a week, go through 5,000 samples, if those samples are available. The tricky thing is how well the patients are identified for having CFS.

We also would like to go back to a much larger population of normal blood donors, at least 10,000, but possibly 20,000. This will be done in collaboration with the American Red Cross also. As you heard from Mike, we are going to be taking part in linked donor-recipient testing, both with samples from the NHLBI repositories and with some linked donor-recipient samples that we will obtain through Dr. Stramer and Dr. Dodd.

Thank you very much. I'll take any questions if there are any.

DR. HOLLINGER:

So, Jeff, this assay can detect both modified polytropic MLV and XMRV?

DR. LINNEN:

Right. With the assay that we have just recently developed, that's the case.

DR. HOLLINGER:

Thank you.

Other questions from the committee?

(No response)

Thank you, Jeff.

We have a final person who has asked to speak. This is Dr. John Hackett, from Abbott. You have 10 minutes, John.

DR. HACKETT:

Thank you. I would like to thank the chairman for allowing me to speak to you. Again, I'm at Abbott Laboratories, a stockholder of Abbott. We do have a relationship with Cleveland Clinic related to licensing of diagnostics related to XMRV.

A little over three years, we initiated studies, because of the concern of another retrovirus, potentially. in the human blood supply. Obviously, there are many questions that are very fundamental in terms of trying to understand causality, and even prevalence and modes of transmission. The key to this will be to have appropriate assays to be able to measure this. Ideally, for these sorts of studies, we would have some serologic tools that would allow us, in a very specific and sensitive way, to reliably detect antibodies to HIV.

Dr. Villinger earlier today showed the studies we initiated in rhesus macaques. Our approach early on is -- we had three primary hurdles. The first is, we didn't know what proteins would be responded to by primates for this virus. Secondly, we didn't have seroconversion samples to work with. Thirdly, we didn't have any real source of bona fide positive control. So our solution to this problem was to create the macaque model.

As he indicated, if you look on the Western blots -- this is showing time after inoculation with XMRV. This was VP62. It came from a prostate cancer patient. You can see that we had gp70 reactivity, which on the Westerns for recombinant are easy to see, p15, as well as gag p30 reactivity. All of the animals responded. I'm showing results from just one here. But this was our very first insight into what proteins antibodies would be generated to in this system.

Similar to what Jeff said, we wanted to move to a system that would facilitate analysis, once we had assays available. In this case, we are working on the Architect platform, which can run approximately 200 tests per hour. We have developed chemiluminiscent assays targeting 15E, gag p70, as well as capsid protein p30.

In terms of these assays, there are two primary formats that can be used. The first is the indirect format, where you have a recombinant antigen or some sort of antigen that captures specific antibodies. Then one comes back to detect those antibodies by the use of an anti-human immunoglobulin. An alternative approach is to use antigen essentially to bridge. You capture the specific antibody with antigen and also detect it with antigen. There are benefits to both systems. I have to say, these are much easier assays to develop. On the other hand, these tend to be far more specific.

To illustrate this, I show here, for the p15E assay that we generated, a distribution on the left of 97 blood donors. These were screened for other blood-borne infections and were negative. In this case, it's relative light units on the y-axis and frequency on the x-axis. Here you had a mean of about 2,300, with an SD of 1,758. Merely changing the format, not changing any of the recombinants involved -- a dramatic difference in distribution. Now you can see in the direct format that we have a mean of 329, with an SD of 114. The impact of moving to that format is that you can discriminate positive and negative samples much more readily.

On the top panel, what I'm showing are 100 blood donors and then 36 Western blot-positive primate bleeds. These are bona fide positives, because we have seen it, and seen it by Western blot. You can see that with the indirect format, you have some overlap here. If we cut it off to call everything that we know is positive, positive, we have only 75 percent specificity.

This came up earlier when Dr. Stoye was speaking. There can be false reactivity in these assays.

to the direct format, that we now have 880 blood donors, including these 100, and the 36 Western blot-positive bleeds. This is a log scale along the x-axis, so you get excellent discrimination between negative population and the positive. In this case, we had one blood donor that was above our cutoff, which is established at the mean plus 16 standard deviations -- so a very conservative cutoff. That individual wasn't positive on Western blot, so we considered it a false negative. I should say, we don't know that with certainty. But we still have very high specificity in that assay.

For gp70, we realized early on, as studies were going on, that we were going to need as sensitive an assay as we could possibly get. I should say, that p15E assay is a very sensitive assay, certainly as sensitive as any HIV assay that's on the market.

If we look at gp70, what we did was use a signal amplification modality in the conjugate, where we have an avidin-biotin system with the gp70. This direct format detects both IgM and IgG, but generates far more signal.

One of the questions, of course, that will come up is, because of the issue raised with the MLVs, in particular with chronic fatigue syndrome, how would these assays perform? The only thing we can do is use other surrogates.

In this case this is a goat anti-Friend murine leukemia virus antiserum. You can see, for the gp70, it titered out at 1-to-16,000, was still positive, 1-to-32,000 in the 15E assay, and 1-to-64,000 in the p30 assay. Another goat anti-envelope -- it's a Rauscher murine leukemia virus -- that titered at 1-to-10,000 for the gp70 assay. This is just showing Western blots, which we have also developed, based on XMRV proteins, as well as recombinant proteins expressed in mammalian cells. I should say that the gp70 assay uses a recombinant-produced gp70 for detection.

I just want to turn to blood donors quickly. We don't have a tremendous amount of data. This shows the 15E assay run on nearly 3,000 blood donors. You can see that we had five of them that came out above the cutoff that we had set. Three of these are quite interesting.

On this slide, on the left panel is XMRV lysate Western blot. These three do have evidence of gag p30 reactivity. This is the gp70 recombinant, and we do see some gp70 reactivity in these three, too. So in this case, we have some samples that do appear to have multiple reactivity against XMRV proteins.

I want to conclude. We have identified the primary markers elicited by XMRV infection. It's interesting that Dr. Bagni essentially has resolved to the same three proteins in their efforts. We have developed three high-throughput assays. Gp70 is by far the most sensitive, 15E next, and then p30. Now we are in the position that we can begin to do other studies. Mike Busch mentioned that we'll be working with him, and Drs. Stramer and Dodd will also be involved with studies along with the Gen-Probe group there. Hopefully, these tools will begin to allow us to determine whether we are seeing transmission of this virus.

I would just like to close by acknowledging my colleagues, as well as Dr. Silverman and his group and Dr. Villinger and his group at Emory.

Thank you.

DR. HOLLINGER:

Thank you, Dr. Hackett.
Questions?

Yes, Dr. Klimas?

DR. KLIMAS:

Are you using both sera and plasma or just plasma in those assays?

DR. HACKETT:

We can use either. It doesn't seem to matter.

PARTICIPANT:

The volume?

DR. HACKETT:

We need approximately 150 µL to load. That makes it tough. But usually that's the issue; there's not enough volume in these samples. That's per assay.

DR. HOLLINGER:

Thank you. Any others?

(No response)

DR. HOLLINGER:

This really exceeds our time for the open public hearing, but I'm going to allow the lady who asked to speak just a minute ago to come up and speak to the committee. Then we'll close the public hearing.

PARTIICIPANT:

Thank you very much. I know this is not protocol.

I just wanted to please ask that -- the idea that "if you are well today, you can give blood," please reconsider that. I appreciate that the AABB and Red Cross maybe followed something a little more rigorous.

I'm a CFS patient. I was diagnosed at Johns Hopkins with CFS and fibromyalgia in 1999. I have had it for 16 years. When you say remitting and relapsing, like Dr. Klimas said, we are not talking about just weekly or monthly; we are talking about hourly. When I first got here, I had rested four days in order to be here today. Slowly, throughout this very long meeting, I'm at a point now where my lymph nodes are killing me from head to toe. I feel like I'm about to get the flu. It's a combination of mono and the flu.

So this morning, I would have been fine to go ahead and give blood. By this point, my cognitive issues are shot. I'm sure other people's are, too. Mine went about an hour or so ago. I couldn't put simple math together at this point.

So please do reconsider that stipulation right now. It isn't effective, even for the small population that knows they have CFS. It's definitely up and down, even daily.

Thank you very much. I appreciate it.

DR. HOLLINGER:

Just a question while you are there. Thank you for your comments. The question has to do with well or unwell when you donate blood. You would be given an educational piece of information, which would say that if you are a CFS patient or have that diagnosis, you should not donate. That would have nothing to do with whether you feel well or unwell at the time of donating. Does that make a difference for you?

PARTICIPANT:

I do believe that would make a difference. I think I like Dr. Klimas's suggestion of having a pamphlet that would be able to explain CFS to the public in general as well. The patient population has been waiting for the FDA, to be able to say that we agree with some of the things that the other blood institutes are recommending right now, and we would like to see the FDA be able to back that up and say, yes, at least for now, while we're working all the science out, let's be cautious and indefinitely defer CFS patients.

DR. HOLLINGER:

Thank you very much.

Dr. Alter, last comment from the floor.

DR. ALTER:

I just want to make a long statement.

DR. HOLLINGER:

That's all right; we can cut you off at any time.

DR. ALTER:

Since Dr. Lo had to leave early, I felt I had to come up and do some defense of him and Judy as well. I think, when a group finds a new agent, they become biased that this agent is real. When another group doesn't find an agent, they become, I think, even more biased that the agent is not real. That leads to this kind of contentiousness.

I think our goal should be not to bring the other side down, but to find the truth. I think the truth will out over the next year, with studies that are already planned.

At this point I concur that we have no evidence for causality. That's going to be very difficult to come by, especially when we are detecting at the limits of detectability and when assay performance is very critical to get equal results. But I still want to counter by saying I think the current evidence for disease association is very strong, even though not universally confirmed.

But it has been confirmed now in at least four studies, two of which were presented today, that either XMRV or a polytropic MLV is associated strongly with chronic fatigue syndrome. A point that I think was misrepresented today: In those labs who do find the agent, it is very reproducible. Judy has found the same patients to be positive by culture year after year. We have found a patient to come back after 15 years and still be positive. So this is not a single, isolated finding. It's confirmed by sequencing. It's reproducible over time.

Dr. Hanson has shown today how critical the assays are. When she tweaked her assay, she went from no findings to findings almost identical to the Lo lab. The diversity is now being confirmed also in the original WPI group. XMRV isn't the only agent even in the WPI lab.

Despite the very legitimate concern for contamination -- I think this is a serious issue -- there have been hundreds of negative controls in the same laboratory that are always consistently negative. An extremely sensitive mouse mitochondrial DNA has always been negative in the Lo laboratory. Lo has done the IPA assay that Dr. Coffin recommended. That is also negative. There just has been no evidence for contamination. Although you could say maybe the negatives could be negative somehow and the positives positive for contamination reasons, it really is not logical that that would be so.

I'm not a molecular biologist. I defer to Dr. Stoye, who is world-renowned in that area. But just as a simple doctor, it seems to me that you have used single-case anecdotal evidence to knock down the various possibilities. I just want to make a case to the committee that you can't -- your conclusion is that anything can happen in assays, and therefore it probably has happened this time. I think using that kind of anecdotal probability is not valid to negate reproducible data from four different laboratories. So at least keep that in mind.

Lastly, I'm not a chronic fatigue doctor, but I have learned a lot about chronic fatigue in the last six months and have spoken to a lot of patients. I'm absolutely convinced that when you define this disease by proper criteria, this is a very serious and significant medical disease, and not a psychological disease. It has the characteristics of a viral disease. It usually starts with a viral-like illness. If XMRV is not the causative agent -- and it may well not be -- there is still need by other groups to look for the next agent which may be the case.

Sorry to take so much time.

DR. HOLLINGER:

Thank you, Harvey.

We are going to close the public hearing and open up for discussion by the committee. I would like to have the questions put up, please.

Agenda Item:
Questions for the Committee

We are just being asked to vote on the first question, and then there is some discussion that will take place afterwards for the other questions that are up there.

DR. NELSON:

Is number 1 one or two questions? The deferral that the AABB talks about is a physician diagnosis, but here it talks about a medical history and/or diagnosis. One could ask symptom questions or other things in the deferral process.

DR. EPSTEIN:

To clarify, we're talking about whether the donor questionnaire should contain a question specific to CFS/CFIDS/ME. The reason we framed it this way is that what we have learned historically about other conditions, such as syphilis or gonorrhea, is that some people react if you ask if you have a history and some people react if you have a diagnosis. Given the ambiguity, we just tried to capture both.

DR. NELSON:

(Off-mic) -- medical history and/or diagnosis.

DR. EPSTEIN:

Yes, that's correct. But let me just be a little bit cautious here. If the committee advises us to go forward with a specific donor question, we might well want to do some cognitive evaluation, behavioral study, to figure out how best to ask the question. But what we are getting at is a broadly framed question that would properly communicate to the candidate donor that they have the condition that we want to defer. The heart of this question is, should there be a question on the questionnaire specific to CFS/CFIDS/ME?

DR. COFFIN:

My answer to this question will depend a little bit on what the meaning of "scientific" is here. If you are talking about the sort of scientific data that we have been discussing all day concerning XMRV, I probably answer this question one way. If you are talking about my general knowledge about this condition, I think I would answer the question differently, excluding the XMRV possibility.

Could we get some clarification on that?

DR. EPSTEIN:

I think, again, without being too precise, we are asking the committee, given everything you have heard and everything you know, do you think there should be a specific donor question? I don't think we are trying to define science. We're all struggling with the quality of the available data. We don't know any more than you have just heard.

DR. HOLLINGER:

I think the issue, at least for me, is when you start to ask a question which is difficult to add to the questionnaire, which is very lengthy anyway, you place a little bit more emphasis on items that you think have more scientific data available. I think that's one of the issues. For me personally, I think the educational material is more than ample right now for what we know about association with disease and transmission and a variety of other things, whereas I think if you asked a question about it, it leaves the impression that we know more than we do. That's how I view these two questions.

Yes, Simone?

DR. GLYNN:

I could not agree more with what you just said, Blaine. I was wondering if we could also maybe vote on a question which supports the provision of educational materials and the self-deferral of donors if they say that they have such a diagnosis or they are not feeling well, rather than -- you can also ask the other question, which is adding a question to the questionnaire.

DR. BIANCO:

I want to support this idea. That's the approach of the AABB recommendation that is being utilized. It's not part of the consideration of the question now. I would like it to be considered, yes or no, that approach. We have over 40 questions that we ask donors on every donation. Those have sub-questions. So things get diluted. Donors, after the third question, don't know where they are anymore. The value of that information that is given to the donor is much more important than simply adding more and more questions.

DR. GLYNN:

I really like what you said about maybe using the information that could be provided to each donor as a way of maybe querying some donors about some symptoms. They may not have been diagnosed, but they may recognize certain symptoms. So if there is more information that is provided, you may be able to also provide more information on the disease.

Again, a question -- since most of them have not been diagnosed, if you ask them whether they have the disease, they are going to say no, because they haven't been diagnosed.

DR. KLIMAS:

I would say this is a tremendous public health potential here. When 84 percent of the people with illness don't know they have it, we have done a very poor job of letting people know that there is an illness out there. Here we have a way to really educate a lot of people, whether or not they have the illness. They might recognize it in a friend or relative. It's a fabulous possibility. I know it's not our primary goal here.

Now, I'm going to say, as a clinician who has treated thousands and thousands of these patients and who has had many, many, many papers about that, we have very robust literature on viral reactivation in chronic fatigue syndrome and immune dysfunction and so on and so forth. From my own knowledge of XMRV, separate from it, there's not a chance that I would let one of my patients donate blood. I have never recommended it. I think it's a bad idea, from infectivity, but also from the patient's own health. They have a 20 percent reduction in blood volume. Why would you give another liter? It's just nuts. But they do, because they are very charitable and kind and giving, and when they are well, they want to do their bit.

So there are a lot of good reasons to want them to defer.

DR. HOLLINGER:

Dr. Rentas?

DR. RENTAS:

I think everyone has summarized what I wanted to say here. If you have taken a look at these educational materials that we are putting out there now -- if I recall, the title is "What You Should Know about CFS and Blood Donation," which we are making available to the donors out there -- to me, that's a much better process at this point, because for 84 percent, if you ask them the question, they are going to answer no because they don't know that they have it. To me, it's a much better process to let them know, "If you have any of these signs and symptoms, you may have CFS. Why don't you go ahead and self-defer and then find out whether you have it or not," than to ask a question, most of the time on a computer, with no one there, that you most likely will answer no.

By the way, I need to put this out. Ever since this policy came out to the AABB, we have called this policy "they don't ask, you tell."

DR. HOLLINGER:

Thank you, Dr. Rentas.

DR. DEMETRIADES:

I think we all agree that there is no evidence about the causative relationship. However, there are some significant at least theoretical concerns about the welfare of the donor and the recipient. In my mind, it makes good sense to have a specific question that excludes these patients from donation. It's not going to affect significantly blood donation. At the same time, we alleviate a lot of fears of the public.

DR. NELSON:

But we're not talking about replacing the educational materials, just adding a question. After a person reads the educational materials, then I don't see anything wrong with asking a question.

DR. BOWER:

I was going to say that I do not believe that we should have this because we think that people with chronic fatigue syndrome can transmit it to others through transfusions. But sitting here and learning something that I didn't know when they talked about all the other commensal viruses they may have and transmit makes me think that it would be a good idea to keep them from donating blood, for their own good and for the good of the recipients.

Analogous to what the best way to do that is, I would like to ask the FDA why they think that the current policy of giving educational material is inadequate and asking a donor-referral question would be better.

DR. HOLLINGER:

Dr. Dodd?

DR. DODD:

I just thought it might be worthwhile reminding the committee that in a time of considerable uncertainty about AIDS, or whatever it was called at that time, Mike Busch reported that a policy of asking would-be donors whether they had a risk factor for HIV/AIDS at that time succeeded -- and he published this in reducing the frequency by about 100-fold in a very high-prevalence area. I think it's important for the committee to recognize that the educational and self-deferral approach does have something going for it. Harvey actually said this, but I think it might have slipped by the committee.

DR. KLEIMAN:

I just want to urge the committee to - because people are saying things that are sort of lumping things together. I think it's two separate issues. Just sticking with the educational material, should it address a diagnosis of CFS or should it, in addition, provide people with a list of signs and symptoms, and let them self-diagnose at the site and make a decision about whether they donate.

I think that latter approach, for one thing, may have a big impact. We don't know how many people have had some symptoms, and it may not be people with CFS. So I think the non-specificity -- I think a diagnosis is -- I'm not a CFS expert, but I think it is supposedly difficult to make a diagnosis, that you really have to interview the person and get five out of eight symptoms or something like that. To rely on a person -- maybe that's incorrect.

DR. KLIMAS:

They have disabling fatigue. That's a very big hurdle. And exercise-induced relapse is a second really big hurdle.

DR. KLEIN:

But I do not think we are going to get disabling-fatigue people -- I mean, if it's that severe, I don't think people are going to come to donate blood.

DR. NELSON:

I don't see anything wrong with combining the question. Maybe the person hasn't had good medical care, but they just got out of bed. It seems like there is a lot of undiagnosed illness. If somebody self-diagnoses, do you want them to donate blood?

DR. KLEIMAN:

I think the point I'm making is that if the recommendation is to educate people and ask them to defer based on their own symptoms, we had better have a very clear way of presenting those symptoms and to differentiate disabling fatigue from somebody feeling that tired on the day or having felt a little rundown in the past week, but not really having -- apparently, you can do that. You can come up with those things. But if we are going to go that route, I think we need help from the clinicians to make sure that we really provide the right symptoms, and not just sort of vague discomfort.

DR. NELSON:

It's voluntary. So if they self-defer because they don't feel well -- I don't know. I don't think that's going to destroy the blood supply, frankly. I think the question should --

DR. HOLLINGER:

Well, it depends on what the material says. If it talks about fatigue and so on, that's one thing.

Dr. Stoye, do you have a response to some of the questions that have been asked here?

DR. STOYE:

I just wanted to say one thing. In Britain, a decision was made to defer indefinitely CFS-positive donors on the basis of their own health, which seems to be what Nancy is saying is at least one reason for doing it. But, in fact, as I understand it, among certain communities, this decision met with derision, because it was said you are just trying to protect yourselves; it has nothing to do with the patients' well-being.

You should think about how you make any decision before you make it.

DR. GLYNN:

So in England do they ask a specific question?

DR. KLEIMAN:

The answer is no.

DR. EPSTEIN:

There is no country that is asking donors a specific question at this point in time. It has been contemplated in some countries, discussed in Canada. But to my knowledge, although there are many that are asking donors to self-defer based on a history or diagnosis, no country is asking a specific donor question.

I think it comes back to Dr. Hollinger's point, which is that we tend to see that as meeting a higher threshold of certainty that intervention is warranted. Again, this can be debated, but that has been the general philosophy. You have to know you're doing some good, in other words.

DR. HOLLINGER:

Dr. Bower, you have been patient. Do you have a question?

DR. BOWER:

You know, I forgot what I was going to ask. Let me think about it.

DR. KATZ:

In fact, there is one country that asks specifically, the Flemish Red Cross.

How many anecdotes does it take to make data? Celso and I are on a group with the European Blood Alliance that monitors emerging infections. When they were queried with regard to the number of deferrals, it's somewhere in the range of what Sue and I are seeing. It's not obvious, unless the Flemish in Belgian have a much lower prevalence of chronic fatigue syndrome -- it hasn't been clear to me that a specific question is, in fact, markedly more effective. But one country.

DR. KEY:

I am not a CFS expert. Neither am I a blood banker. I have to ask, if five years from now the link between XRMV and CFS is shown not to be the case -- there is no link causally -- and this is an indefinite deferral, what happens at that point? We heard most of the data in terms of transmissibility, I think. But we are hearing now that, particularly in the U.K., it's to do with the donor health as well.

What are we being asked to vote on here? Are we being asked to vote on both of those, one of those? Do they stand alone, individually?

DR. HEWLETT:

I just happen to be up here at the microphone. I was going to make a comment about Lou Katz's information about the Flemish Red Cross. It's our understanding that they already had in place a question a while back and it was really not connected to the identification of XMRV. I just wanted to make that point.

No country has put any questions in place as yet as a result of this finding of MLV and XMRV.

DR. BOWER:

Just to answer Dr. Key's question, at least this committee member is looking at this aside from the XMRV. The data on that is too all-over-the-place to really allow me to link that to chronic fatigue syndrome. I'm just looking at it as, should someone with that diagnosis be allowed to donate?

DR. HOLLINGER:

Prerogative here. I think, unless there are some burning questions, we have talked back and forth here. I think we'll go ahead and vote on this question, unless somebody has a real burning -- yes?

DR. BIANCO:

Real burning question. The real burning question is, is there an opportunity for this committee to recommend the approach that AABB is recommending of providing educational materials to the donors, without asking a specific question or not? These questions say -- I can't vote, but if I answered no to this question, that doesn't mean that I don't agree with the AABB is recommending. That's missing there.

DR. HOLLINGER:

To me, I think it's obvious. The AABB already has educational material out there. We're assuming that that would remain the same. Yes, Jay?

DR. EPSTEIN:

FDA pays a lot of attention to voluntary standards, and if we object, we make our objections known. We do not object to the AABB voluntary standard. It's true that we could establish that as an FDA recommendation. I don't object if the committee wants to weigh in on that point. But we do see value in the AABB current practice.

DR. GLYNN:

Could we also recommend that maybe more data be collected? I personally support the AABB recommendation. Let's say that you go ahead and gather data at the blood banks -- some of those data are being collected, but maybe you could collect additional data. If you ask the question, in a particular research study, how many patients would have self-deferred, you could gather additional information to inform us about exactly what's happening with just the self-deferral and the provision of the information. Would that be possible to do?

PARTICIPANT:

(Off-mic)

DR. GLYNN:

I don't have any money -- not personally, that's for sure.

DR. HOLLINGER:

Okay, I think it's time to answer this question. The question is straightforward. It's really about asking a question of donors who come into the blood bank about a medical history and/or diagnosis of CFS as a basis for indefinite deferral. It's about a question, not educational material. "Yes" would be that you favor asking a question; "no" would be that you don't favor it, that you think -- in my opinion, what it means is that you favor the educational material which is currently being used.

DR. BOWER:

I want to make sure that we understand that, because you threw in the educational material. It sounds to me like a yes vote means that they put a question, "Do you have a diagnosis or history," on the questionnaire versus a no vote, which is the status quo, that they proactively provide information and allow them to self-defer. DR. HOLLINGER: Yes, that is how I would interpret it.

You see your blinking lights before you. You can vote yes or no or abstain.

(Vote)

Before we look at these, Dr. Bianco, how would you vote?

DR. BIANCO:

I would vote no. I favor the educational material.

LCDR EMERY:

The committee has voted. We have 9 that have voted yes, we have 0 abstentions, and 4 that have voted no.

Dr. Coffin has voted yes.
Dr. Judith Baker has voted yes.
Dr. Klimas has voted yes.
Dr. Bower has voted no.
Dr. Troxel has voted yes.
Dr. Nelson has voted yes.
Dr. Hollinger has voted no.
Dawn Aldrich has voted yes.
Dr. Trunkey has voted yes.
Dr. Demetriades has voted yes.
Dr. Rentas has voted no.
Dr. Key has voted yes.
Dr. Glynn has voted no.

DR. HOLLINGER:

Thank you. So we have a vote.

Let's go on with the second question, which again does not require a vote, just comments. The question is, please discuss whether the scientific data support asking donors -- it's the same question as the one before, but it has to do with prostate cancer as a basis for indefinite deferral.

Comments?

We're not going to vote on this, so feel free to give your comments.

DR. BOWER:

My comment is that looking at the available data, there is no association with prostate cancer after blood transfusion. If you look at the studies that have looked at XMRV in prostate cancer patients, they have not been able to find it in the blood. I believe that having prostate cancer does not put you at risk for a transmissible disease, so I would say no.

DR. HOLLINGER:

I have seen one report from Japan that apparently found virus in PBMC of one or two donors, I think, out of four. Does anyone else had any data that they would be able to talk to us about, about finding it in the plasma or PBMCs of patients with prostate cancer?

DR. TRUNKEY:

I don't have anything in that, but the most positive things I heard today were the three studies that were presented. One was to go back and look at blood samples in a repository, repeat that, and then try to link it to the things that we are talking about here. The second one is the effort to look at other antigens within the virion, which I thought was very good. Then the macaque studies I thought were very good, particularly as it pertains to prostate cancer, because he showed us the virus is there in the prostate in these macaque monkeys.

I think we have an opportunity here in the next year or two to be able to answer this question. And I think we should wait.

PARTICIPANT:

Infectious virus and antibody have been found in prostate cancer patients. I have found it.

DR. HOLLINGER:

Infectious virus in blood or in the tissue?

PARTICIPANT:

From the plasma and PBCs of prostate cancer patients.

DR. HOLLINGER:

And this is published?

PARTICIPANT:

No, it's not published.

DR. COFFIN:

There is a small-scale, 100-patient study in process within the NCI to try to dissect this issue in more detail, in at least the small sample of patients being treated at the Clinical Center. That should provide us with some more information on this issue within the next year.

My yes vote in the case of CFS was not based on the XMRV connection at all. On that basis alone, I would have voted no. But it was based on the evidence that I heard that matches my intuition and that of other people that there could well be an infectious agent here. It might be XMRV; it might be something else. For that reason, that kind of caution is warranted.

There's no such evidence in the case of prostate cancer. There's no evidence, anecdotal or otherwise, for any kind of a predisposing infection or anything that I'm aware of. I thought the discussion we heard earlier on the subject from one of the public presenters was very much on the mark on this issue.

DR. HOLLINGER:

Dr. Hewlett?

DR. HEWLETT:

I was just going to respond to your question about whether PBMCs were found to be positive in prostate cancer patients. We heard from Frank. There are some published studies. The one that I had on my slide is actually by Fischer et al., from Europe, where they looked at 40 PBMCs from prostate cancer patients. They were all negative.

As I understand it, the CDC presented some data at the CROI meeting last year where they had looked at some prostate cancer patients, found them in the tissue, but the PBMC was negative.

So that's what we have to date. But you're right, it might be worthwhile doing a bigger study to get more information on that.

DR. NELSON:

Where the virus was found in tissue of patients with prostate cancer, was it early and localized? Was it when the tumor was localized or was it after the patient had either metastatic or -- in other words, when is the risk?

DR. KLIMAS:

Those are mostly the biopsy-for-diagnosis samples. So it was pretreatment and, I'm sure, at all stages of illness.

DR. COFFIN:

In the study published from Ilo Singh's lab, it was found in all stages, but there was a correlation of frequency of detection with the Gleason grade of the tumor -- not necessarily with the stage of the tumor, but with the grade of the tumor.

DR. NELSON:

So the virus could have been secondary to the tumor.

DR. COFFIN:

That is always a possibility.

DR. HOLLINGER:

By the way, I forgot to mention that Dr. Stephan Monroe is in the audience. He is a guest from the CDC. He's not a voting member.
Do you have anything to add to this discussion?

DR. MONROE:

Just to clarify the CDC data on the prostate cancer, I believe there were three samples in that study that were positive, of which two had matched PBMC, and those two PBMCs were negative for DNA. It's very small numbers.

DR. HOLLINGER:

Thank you. If you have any other comments somewhere down the line, please feel free to jump in.

Go ahead.

DR. SHOCKMAN:

Gerry Shockman, from Abbott.

I just want to mention to the committee that the original study by Urisman and Silverman, as well as the Danielson and Kimata paper, really were suggesting that the XMRV was not found in all prostate cancer, but was in hereditary or familial cases. The Danielson paper actually looked particularly at familial cases, where there were close relatives. So when you think about XMRV and prostate cancer, I think you have to be a little careful and not paint this with a broad brush, and think about, more specifically, the issue of familial or hereditary forms of prostate cancer.

DR. DEMETRIADES:

Coming back to Dr. Nelson's comment, although there is no published association, is it possible that perhaps in situ cancers are very different from more advanced cancers? That's my concern. I think they should be excluded.

DR. HOLLINGER:

Say that again, please.

DR. DEMETRIADES:

It is possible that blood donors with an early in situ cancer -- it's fine, it's safe. But patients with more advanced cancer maybe could transmit the disease.

DR. HOLLINGER:

Dr. Rentas?

DR. RENTAS:

Thank you. I just wanted to say that, as opposed to CFS patients, I don't think there is a blood collection facility in the U.S. that I know of that doesn't cover cancer as part of their questionnaire already. This is already covered. If you do have a history of cancer, to include prostate cancer, you will be deferred from donating.

DR. NELSON:

Even if successfully treated?

DR. HOLLINGER:

My understanding is that if you are asked the question and you have had prostate cancer and you have been free for a year, off therapy, then you are able to donate. You can donate at that point. If I'm wrong, would the blood banking community let me know?

DR. NELSON:

This says medical history. If we agreed with this deferral, that person would be deferred, if they had an in situ and had a prostatectomy.

DR. HOLLINGER:

Simone?

DR. GLYNN:

I just want to remind everyone that the data we have is that there is no association that we know of between prostate cancer and a transfusion history. Neither do we have any data -- it's very little data, but the little data that we have does not support an association between chronic fatigue and a history of transfusion. So you don't have that particular association.

DR. NELSON:

Yes, but here the concern is not so much whether the prostate cancer resulted from a virus transmitted by transfusion, but whether the prostate cancer patient maybe has a superinfection of their tumor and is infectious as a blood donor. We're talking about deferring them. I'm not sure it's the same.

DR. BIANCO:

There are huge studies done in Sweden that have analyzed years of transfusion recipients and any association with cancer, and they have failed to find that association.

DR. HOLLINGER:

I guess the question would be, though, Celso, if some of these studies, for which we'll be waiting on the results -- if you do find that it's positive in the plasma and/or in the PBMCs, then I think you are probably caught at that point in asking the question again or using educational material, whatever. I'm not sure how you get away from that. I'm not talking about causality or anything.

DR. BIANCO:

We will be back here talking about it.

DR. KLIMAS:

Just a comment. In Frank's data -- I can't speak for where the samples came from -- most of these biopsies are time at a time when someone is acutely ill, and so they are going to be more viremic, one would presume. Someone who is post-prostate cancer -- that's what you want. You need that sample, to be able to see if there is any plasma or cellular infection in that sample. I don't hear anyone saying they have that for us.

DR. HOLLINGER:

I think what we have here is that we are waiting for more data. There are some studies that are going to be pursued with that information.

Let's go on to the third question.

The third question is, please comment on the scientific evidence that would be needed to justify a policy of donor testing for infection with MLV-related human retroviruses. In particular, should donor testing be considered in the absence of confirmed disease causation?

Comments. One thing that I have not seen that I would like to see is testing in other patient groups that are immunosuppressed or even with cancer. We have it in breast tumor. We have a small study there. We have another small study in the respiratory infections and so on. But I have not seen any other studies looking at detection of this virus in immunosuppressed groups. I personally would like to see that kind of information.

DR. BOWER:

There's a negative study in HIV.

DR. HOLLINGER:

That's right, in the HIV group.

DR. STOYE:

In one of the negative studies for CFS, the control group included some transplant patients.

DR. HOLLINGER:

Thank you. I appreciate your telling me that.
Other comments about what kind of evidence you would need for such a policy in terms of donor testing.

DR. BOWER:

I will just say, at a minimum, it would be consistent evidence of association with the various studies that are going on, especially the collaborative studies, like the Phase III and the other studies that Mike Busch talked about.

DR. HOLLINGER:

So you would need to see transmission. You would need to see infection. So transmission, then some sort of infection or disease causation preceding that --

DR. BOWER:

At a minimum, just an association. I think I would like to see disease causation as well.

DR. DEMETRIADES:

I do not think the existing evidence can support routine testing.

DR. COFFIN: I completely agree with that. I think what's needed here, really, is one of many different possible kinds of tests. It's hard to say exactly what the test is. But the critical thing is to get some kind of consensus out of it.

Does you have a test that -- this is what happened with HIV, for example. Soon after it was discovered, it did not take very long before there was a consensus that this was there. As far as the more subtle issues of causality, pathogenesis, it took a much longer time to iron out. But once people knew what to look for, everybody could find the virus.

We're not at that stage with this yet. We have to be there before we can even begin to move forward to ask questions about causality. I think, as Jonathan said at the XMRV meeting, none of us came into this trying to disprove the idea that this virus -- none of us came into these studies looking for negative associations. But that's the way the data that many people have found have turned out. So we are still lacking the kind of scientific consensus that there is really infection in people, much less what the extent of that infection is, and much less the possibility that it's causing CFS or some other disease. We simply have to start by getting a way to move forward by having consistent assays and consistent studies that just give the same results from one lab to the next. That's still not the case.

DR. MIKOVITS:

(Off-mic)

DR. COFFIN:

It has been a long time. Until your publication, Judy, the field was very, very quiet, I have to say.

DR. MIKOVITS:

In HIV/AIDS, that was five years after the first publication of the Science paper isolation to where groups were agreeing on people who were infected. We didn't have a PCR assay in 1982, at the first publication. So we isolated virus and looked for serology.

DR. NELSON:

It seems to me that one question that probably will be answered, hopefully, in the not-too-distant future is whether or not this disease is transmissible by transfusion, by looking at these repositories. That's one of the values of these repositories. I think that question can be answered. Obviously, if that is negative or if the risk is very low, then that would make it less important to worry about screening donors.

But if there is clear evidence of transfusion, then the endpoint of that -- now, it seems possible that some of the people who received transfusions and got infected 20 years ago -- that they might have had an outcome by now. It's possible. They might have had CFS or some sort of outcome from the infection. That's the value of having these cohorts and having samples from the cohorts.

DR. HOLLINGER:

There are several studies which have followed patients -- for example, our study, initially -- which have looked at patients 25 years later that includes the TTV study, as well as the NIH study, as well as the VA study -- in which patients were looked at again 25 or 30 years later. Those samples are available, if you find some positives, to look at those individuals later. There are several studies, Rh studies in Germany, in which females acquire disease. They have been following them for 35 or 40 years or so. I think that would be important to look at as well.

DR. EPSTEIN:

Question 3, if I could perhaps communicate a little bit of what's troubling the FDA -- I agree with Dr. Nelson that the studies that are being launched will probably tell us in the next year or two whether there is transfusion transmission of the virus and infection. But proof of causation may take a lot longer. What we are really trying to get at -- these viruses are sort of in a bad family. When you look at what they do to the mammals that they infect, you have neurologic complications, immunosuppression, and cancers.

The question is whether that background is sufficiently compelling that we should be precautionary or proactive about donor screening, if transmission is shown. Or should we actually wait? I think the arguments can go two ways. For example, with simian foamy virus, which we discussed with the Blood Products Advisory Committee, we had pretty much the same set of questions: Is it transmitted? Is there disease association? There was one look-back study that didn't show transmission, even though we think transmission might still be possible. But the compelling argument for the BPAC was that there was no apparent disease association.

Here the issue, I think, is a little bit differently framed. As I say, in other species, there is clearly disease association. So should we be waiting for causation or not? I think that's the perspective we are trying to get clarified. We are trying to anticipate if studies of transmission show transmission, but we still haven't demonstrated disease causation. Do we act or don't we act? You know, we can wait and cross that bridge in a year or two. We don't have to answer it today

DR. HOLLINGER:

We won't do it tonight.

DR. NELSON:

But wouldn't some of the repository studies -- if the recipients had a transfusion-transmitted infection in 1983 and a higher proportion of them ended up with some sort of outcome, it's conceivable that we might be lucky -- or if they had no outcome. Those data would be useful.

DR. KLIMAS:

Just one clarification. Probably Judy can do this better than I, because she and Dan Peterson reported this very large case series of mantle cell lymphomas in chronic fatigue syndrome patients. It's a kind of tumor that most oncologists don't see in their entire lifetimes, and yet there is this very large series. I know Judy has done a lot of work with that.

I myself have seen a number of lymphomas in my patient population, usually EBV-associated lymphomas. We don't know what the coinfection roles might be. So I am afraid of the oncogenic potential of this type of virus in the population.

DR. COFFIN:

With all of the models for oncogenesis by these viruses, that would be the best possible scenario, in fact, because it's really easy to show. If what is happening in a patient with a disease like that is the same as what happens in a mouse or a cat or any other model of disease that is caused by these viruses, every single cell and tumor that's going to have a provirus is going to be sitting next to a proto-oncogene. It's a real smoking gun that is easy to detect. So study of diseases like that could provide by far the best evidence in a real hurry, if that was possible.

I would agree, though, that if, in fact, we accept that the virus is present in some people, in a fraction of people, and not in everybody, and if we have a good, validated assay that there is consensus on that shows that, and if that virus is being transmitted by blood transfusion, I would consider it highly prudent to test for and decline blood that was likely to be contaminated with this virus, even if we don't have proof of causation of this disease. I think there are too many other things it could be doing that are bad to take that chance.

DR. MIKOVITS:

I would just like to comment on Nancy's comment. We have a very large group, 30 percent of the cohort that Dan Peterson has studied across the United States, with both CLL and mantle cell. With one particular individual, we could isolate virus from 1984, before he was diagnosed with CFS in 1988 and 10 years before he was diagnosed with mantle cell in 1999. He succumbed to mantle cell in 2008. We have samples where we can isolate the virus throughout the disease and a cell line where every single cell is infected with the XMRV, and not EBV or any other virus that is known.

We have a number of cases like that who have gone on. At least one -- you should have received written testimony from a gentleman who couldn't be here, who developed the disease after surgery and a blood transfusion, a JD/MD who couldn't work any longer. There is mantle cell in his family as well.

DR. HOLLINGER:

Thank you.

Let's look at the last question, the final question: Assuming that testing is warranted, please comment on the potential utility of nucleic acid tests and/or serologic testing of blood donations to ensure safety of the blood supply from transmission of MLV-related retroviruses.

Any comments from the group?

DR. COFFIN:

My answer is yes.

DR. HOLLINGER:

And it looks like these tests can be validated, hopefully, in time and would cover a broad spectrum.

DR. NELSON:

This question depends, really, on the sensitivity and cost and so on of these assays. Right now we don't know for sure.

DR. HOLLINGER:

That could reduce the blood supply by anywhere from 1 to 8 percent?

DR. NELSON:

Depending on the number of false positives, I guess.

DR. HOLLINGER:

Any other comments from the committee, besides being tired?

Jay, do you have anything else that you want from us?

DR. EPSTEIN:

Of course, these technologies will continue to evolve. I think what is concerning the FDA is that when you look at the current state of the art of NAT tests, there is a lot of variability, we seem to be at some limit of detection, and the procedures would require types of sample processing that are not currently feasible in the donor-screening setting. Also there is the possibility that over the course of infection, titers in the blood may go down; the game may just be in the tissues.

So what we are really trying to think about is, is it a better target to pursue serologic testing? What we have seen so far is that the yield or positivity rate in serologic testing with current, state-of-the-art tests is seemingly lower than NAT in these high-risk cohorts. So what we are really looking for from the committee is, is there a basis to advise us where to invest effort? Should we be trying to facilitate NAT versus serology? Should we just be neutral and try to develop both, recognizing the logistic complexity of NAT.

DR. HOLLINGER: I think one of the problems with looking at NAT in plasma and PBMCs and so on -- in so many cases, you have to do a lot of manipulations. That's not going to be possible in the blood community – ultracentrifugation, putting it in culture to amplify it, doing activation. Where you are collecting PBMCs, you are not going to do that, I don't think, in the blood bank. This, to me, is one of the major issues that has to be resolved, as distinct from serology, which is a simple enough test to do. I think that becomes a real issue, and how sensitive that's going to be and which one is going to pick up the most with a good throughput.

DR. BOWER:

Right. Obviously, getting a test that is consistent and one that works is what you need to do. It seems like if you had all three that were working well, serology would probably be the best, because it would be the easiest to do. At least I assume we are looking for chronic infections and we not trying to pick up incident infections for this -- although I don't know, because I don't know exactly what the incident rate is of chronic fatigue syndrome.

DR. HOLLINGER:

Yes, Dr. Coffin?

DR. COFFIN:

My answer wasn't entirely flip. In the case of HIV, if you think about that as sort of a model, both of these have their place. It is well understood that serologic tests don't detect early incident infection, as you point out, and they have high false-negative rates, particularly in the context of widespread screening, requiring repeat testing with other methods. Nucleic acid-based tests are more cumbersome and more expensive to do on a broad basis, but provide a greater level of sensitivity and detection of incident infection.

I think we don't know how this is going to play out until we understand much more about the biology of the virus, if there is a biology of the virus. So we really can't answer that question yet. I think this has to be pushed forward on all possible fronts in these areas, and perhaps even others, if somebody can think of another kind of test, until we know we are really beginning to get somewhere and we can begin to bore down with that particular set of studies.

The two companies that presented, Abbott and Gen-Probe, are to be applauded for the amount of resources that they are putting into this. They are taking a real risk, but they have the potential of bringing us the best possible assays to be used for this kind of screening.

But we still don't know where we're going. We're casting around in the dark right now.

DR. HOLLINGER:

Thank you.

I don't see any other burning comments from the committee here. I do want to thank both the audience and the committee for your patience. This has been a long day, and we are very appreciative of your staying around. I think we have learned a lot about this disease.

Tomorrow morning, 8:00, we'll get started. Hopefully we'll finish on time tomorrow.

(Whereupon, at 7:15 p.m., the meeting was recessed, to reconvene the following day at 8:00 a.m.)