very recently we have tried to push these animals, giving them extremely high doses in the range of 1014 to 1015 per kilo, and we do not increase the proportion of integrated genomes.

The proportion of transduced cells with integrated genomes is small and most integrates that when we have actually molelecularly analyzed them are 1 or 2 copy genomes.

[Slide.

The clinical trial objective is to test the hypothesis that AAV mediated liver-directed gene transfer is safe; characterize the human immune response to the transgene product and to the vector; determine whether germline transmission of vector occurs following hepatic administration; and determine dose capable of producing clinically relevant factor IX levels in the blood.

[Slide.

It's a Phase I open-label, dose escalation safety trial of AAV Human Factor IX administration by infusion into the hepatic artery.

[Slide.

The vector is infused into the liver via a balloon occlusion catheter placed in the hepatic artery, and Factor IX protein is administered

before and follow the procedure to cover the patients from any type of bleeding.

Subjects are observed for at least 24 hours

[Slide.

This is the dose escalation plan of the trial as it is written. The dose in vector genomes is 2 x 1011 per kilogram. The observed levels in mice is somewhere between undetectable and 1 percent.

Importantly, is that when you get into the second cohort, we were at a dose of 1 x 1012 per kilo, and in dogs that were given a similar, not identical vector, levels in the range of 4 to 12 percent are achieved.

These levels of Factor IX would result in a substantial improvement in the clinical course with the individuals going from a severe phenotype to that of a much milder phenotype. So this would be somewhere in an efficacious range, so the point is that at doses within this trial, we are at efficacious doses in a dog model of hemophilia.

[Pause.]

DR. KAY: I am really sorry. There was a mix-up about transferring the slides, so I

apologize.

This was just an introductory slide about hemophilia, basically that it is a very well understood disease and with sustained levels of 1 percent, you can get a therapeutic response, and we do have very good animal models which are the dogs.

Now, this is basically what I said, that we have actually been able, we and others and more recently Kathy High's group, has gotten reasonably high and therapeutic levels of canine factor IX in dogs reaching 4 to 12 percent. I won't go through this again

[Slide.

This is just a photograph of a patient who is being treated here. As I said, it is through the hepatic artery and they go into the invasive radiology suite. A catheter is inserted into the femoral artery and it is cannulated into the hepatic artery, which can be followed by fluoroscopy here, and then the vector is placed on an infusion pump, as shown here, and then administered at a specific rate into the patient

[Slide.

Now, the first subject that was treated is a 63-year-old male with severe factor IX

deficiency. Status/post bilateral knee replacements 5 years prior to the procedure. He is HIV-negative. He was HCV-positive, but his HCV viral load by PCR was negative on multiple occasions several years apart. Per our protocol, these patients are considered to have spontaneously cleared HCV, and do not require liver evaluation before being enrolled into the trial.

He is the father of 3 and he has a grandson with hemophilia.

[Slide.

The first procedure was done in August of last year. He received 2 x 1011 vector genomes per kilogram. No complications. He was discharged home to his referring hemophilia treatment center after five days

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This is a summary of his clinical data baseline before the procedure and afterwards out to week 24. The important point here is that his CBCs have all been within normal limits including platelet counts, which have been an issue with some of the adenovirus trials

[Slide.

His liver function studies and prothrombin

times have also remained normal, as shown here. His ALT and AST are normal, and they remained normal throughout the 24-week period for which he has bee monitored.

So the hepatic administration of this vector in this patient did not appear to have any liver injury

[Slide.

The coagulation data for this first patient is shown here. His factor IX levels have basically remained at a subtherapeutic or nontherapeutic level. This basically is background. Remember that these patients do treat themselves.

The important issue here, too, is that this patient did not have detectable factor IX inhibitor by Bethesda assay.

[Slide.

One of the aspects of the protocol is to monitor the different body fluids for vector shedding and, of course, the reason why we are here today. This just is a very simplified diagram of the PCR assay that is done by Deb Leonards' group at the University of Pennsylvania

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This shows the actual sequence of the vector and the PCR primers are depicted here as a control for the PCR reaction itself. Some of the samples are spiked with very small plasmid numbers of a second vector that has the same sequences for the primers, but there has been a deletion of 97 base pairs, so one can distinguish between the spiked copy, if you will, and the vector copy

[Slide.

This just shows an example of one of the gels of this analysis here. This is the baseline sample here. This is the spiked sample below, and this is day seven of a body fluid where you can see both the spiked and the actual vector band shown here. So this gives you an idea of the PCR studies. Some of these will be discussed again in more detail with some of the preclinical studies.

If we look at the vector sequences by PCR, in the different body fluids here, in the first patient, again, we see transient vector DNA up until week 2 in the serum, transiently for a couple of days in saliva, there was none in urine and stool, and white blood cell pellet was done at week 12, but that was negative

[Slide.

This is what was somewhat of a surprise to us based on dog studies we had done. In fact, when we did look at his vector DNA in semen, we did find that there was DNA present in his semen, but it was transient and it slowly fell off over a period, and after week 12, has remained persistently negative.

Now, these samples are performed in triplicate in 1-microgram DNA samples. When we did get positivity in these first couple of samples, we went to a fractionation procedure to try to fractionate out the motile sperm fraction from the seminal fluid sample and the pellet.

Now, in this motile sperm fraction, we were only able to get 220 nanograms of DNA, so it wasn't the 1 microgram, but this amount of DNA was PCR-negative in this individual.

I also want to point out the sensitivity of the assay is less than 1 copy per 30,000 haploid genomes or, in other words, 1 copy per 30,000 sperm.

Now, as a result of this result, we did make some changes in the consent form related to the issue of informing the patients about this result, and basically, what it says the study subjects shall be adult males who are 18 years of

age or older.

The first patient treated under this protocol was very shown by very sensitive techniques to have vector in his semen for as long as 10 weeks after treatment. Although the vector was not found in the sperm fraction, the significance of this finding is unclear, and all patients are strongly urged to use barrier birth control devices, condoms, until the patient is informed that semen has been clear of vector for at least three months.

The investigators will notify you when it is safe to stop barrier methods of birth control. The consequences of gene transfer, the germline cells are unknown, but could potentially result in serious birth defects or fetal death or other unanticipated health consequences, such as cancer, in the offspring due to the disruption of normal genes by the transferred DNA. If you are considering having children in the future, it is recommended that you bank sperm before beginning the procedure to ensure a source of sperm that is free of contamination with the vector.

The reason for storing semen is that it is possible that if the sperm cells do take up the

vector during the procedure, it may or may not result in life-long changes to the sperm. The investigators will provide you with information on sperm banking and this one is for Stanford at Stanford University or at your home institution. This opportunity will be provided to you at no additional expense.

So the point here is that we urge the individuals to undergo a barrier contraception, we talk about the risk in this first patient, and the fact that we will sperm bank in case they are considering or uncertain about future childbearing.

Now, because of this issue of finding, at least in the first patient, transient AAV vector sequences in the semen, we amended the plan to address this issue of inadvertent germline transmission, and the protocol was changed, so that semen collection was done as a baseline, and then at weeks 1, 8, 12, 16, or possible more.

Now, the idea was, and the plan is, that beginning at 8 weeks, the sample is then fractionated and total semen and motile fractions are analyzed for vector genomes by PCR. If the 8-week motile sperm fraction is negative, we would be allowed to proceed to the next dose cohort. All

subjects to practice barrier contraception until three consecutive monthly semen samples are negative.

So, although we will test and fractionate through week 16, the question is we continue if there haven't been three successive negative semen samples

[Slide.

Subject 2 was a 48-year-old male with severe hemophilia B. He had a bilateral knee replacement in 1999 and elbow replacement in 2001.

He is HIV-positive and HCV-positive. He underwent a liver biopsy and was shown to have minimal fibrosis and based on criteria in the protocol, was allowed to be included in the study.

He had a non-Hodgkin's large cell lymphoma in 1986, was treated, had a relapse in 1996, and was treated and he is on medications for his HIV

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The procedure was performed in January, the end of January of this year, received the same dose as the first patient. No complications. Went back after 7 days

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Patient 2, like Patient 1, had totally

normal LFTs, no elevations related to the vector

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Renal function, not shown with the first patient, but were also normal in the second patient

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Again, the CBC including the platelet counts were normal. There was no elevation with vector administration

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Now, with the second patient, again, we see no evidence of inhibitors, and we have also noticed that there is a question of whether there is any detectable factor IX in this patient. The week 8 and week 12 samples were obtained at least 14 days prior to factor IX administration, and there are some low levels of factor IX here detectable, but again it is unclear whether this is really and truly from gene transfer. I just wanted to point out that this is the data to date. So it is still questionable

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Now, when we looked at his body fluids, the saliva was positive for a slightly longer period of time, up to one week. His serum was also positive up to four weeks, which again was two

weeks longer than the first patient.

Unlike the first patient, we did see transient positivity in the urine, but only out until day 2, and he also has had some positive stool samples, as well

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Now, this is where we are with the semen analysis for the vector DNA. He has remained positive up through week 14, but let me talk about the total semen first.

The total semen, the signal of the PCR has started to diminish, similarly to what we have seen in Patient 1. If you remember Patient 1, he was persistently negative after week 12, and the week 14 sample, which we just obtained this week, although it was positive, the signal appears to be weak, so it appears to be going down in concentration, although this is not an absolutely quantitative assay.

Now according to the protocol, we were supposed to fractionate his week 8 sample into the fractions that I discussed earlier, to look at the motile sperm fraction, but it turns out that this individual has ejaculate volumes that are well below half a ml. When the sample went to the lab,

it has got to be fractionated within about 30 minutes or so, and when they got the sample, the lab said, you know, based on our SOP that we have, and the one that is provided in the protocol, this volume is not adequate to fractionate, so it wasn't fractionated.

Well, we went back, and after discussions with FDA and our colleagues, we realized that there are standard operating procedures in these clinical laboratories to fractionate low-volume ejaculates, and this then was attempted on the week 14 sample.

But unfortunately, the DNA recovery from this week 14 sample was such that it would only be possible to run triplicate samples of 300 nanograms per ml, and based on our changes in the protocol, which we have just sent to the FDA, this would be a fractionated sample that we would not analyze. So the fractionated sample with 300 nanograms in it was not analyzed by PCR.

It has turned out that although it is simple in theory, it has been difficult, a little more difficult than we had anticipated doing these fractionation procedures and getting the kinds of DNA recoveries that one would want.

This individual has supernormal sperm

counts so although his volume is low, it appears that spermatogenesis in this individual appears to be normal because his counts are well above normal.

It also turns out that there are lots of rules and regulations in the labs that do the fractionation. In fact, we are learning that many of these labs are not allowed to fractionate HIV-positive samples, which has also led to some of the difficulty in getting these specimens fractionated at will.

So based on this, we have added new exclusion criteria. We realize that this individual has an issue with ejaculate volume, but with normal sperm counts, that is very, very rare and unusual, but because of this in this patient, we have added an additional exclusion criteria to a revised protocol.

First of all, we state in there that an exclusion issue are related to patients who are unwilling to provide required semen samples, and patients that are unable to provide semen samples of adequate semen volume, which we define at 1 1/4 ml sperm count, and we define the cutoff at 20 x 106 sperm per ml, and with motility of greater than 50 percent. Again, this was based on the data we

have obtained from this Patient No. 2.

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So, in conclusion, I can say that Subjects 1 and 2 have tolerated the procedure well, vector DNA is present transiently and total semen from Subject 1, not present in the motile sperm fraction at week 3, albeit the sample that was analyzed was 220 nanograms, not the desired 1 microgram.

We have much limited data in Subject 2 although the signal is going down, we still haven't detected a sample that has been negative, and currently, based on what has been approved, that the enrollment of the subjects at the mid-dose proceeds only if Subject 2 shows absence of signal in the motile sperm fraction.

So, in summary, what I would like to say is that clinical studies demonstrate safety and long-term efficacy of AAV factor IX in the liver in the large animal model of hemophilia. We think that this is really the impetus to move forward.

The initial clinical studies indicate that this gene transfer strategy can be safety translated into human subjects, and we strongly believe that the completion of the Phase I study is required for valid risk-benefit analysis of the

strategy.

We would like to present a proposal to you of what we would see as a reasonable route of moving forward, but before we do that, there will be two additional speakers who are going to present the preclinical data studies that have been done to try to address this issue, what has been done, the data to date, future studies in a number of different animal settings.

Thank you.

DR. SALOMON: Thank you very much.

We won't have any questions until after the second speaker.

This second talk is from Linda Couto of Avigen entitled Safety Studies to Support Intrahepatic Delivery of AAV.

Safety Studies to Support Intrahepatic Delivery of AAV

Linda Couto, Ph.D.

DR. COUTO: I am going to describe a series of preclinical studies that were performed to evaluate the safety of delivering AAV to the hepatic artery

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We have used five different species -

mice, rats, dogs, rabbits, and monkeys to assess the toxicology and biodistribution, but today, I am going to limit my talk just to the biodistribution studies that are relevant to inadvertent germline transmission

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I am going to summarize the studies in rats, dogs, and monkeys, and then Valder Arruda is going to present some more recent data in rabbits, which appear to be probably the best model for studying inadvertent germline transmission.

However, before discussing the biodistribution data, I just want to point out that in all of these five species, we haven't seen any toxicology at doses up to 1 x 1013 vector genomes per kilogram, which is 50-fold higher than our starting clinical dose.

This is the biodistribution study that was performed in rats. In this study there were five groups of animals. One group was treated with the excipient. One group was treated with an AAV null vector, which does not contain a transgene. Then, there were three groups of animals that were injected with increasing doses of an AAV factor IX vector from 1 x 1011 per kilogram to 1 x 1013 per

kilogram.

So what you can see is that at 50 days post-injection, we saw a good gene transfer to the liver, so at the low dose we were seeing about 1 copy per 60 cells in the liver, and at the high dose we were seeing about 1 copy per 1 to 2 cells.

At this time point, we also did see vector dissemination to the gonads at least in some of the animals. At the low dose we didn't see any dissemination, but at the high dose we saw about 1 copy per 1,700 cells, so this was about 1,000-fold lower than the gene transfer we were seeing in the liver.

At this time point, we were also seeing vector in the blood, however, by day 92 post-injection, we no longer detected any sequences in the blood, and the level of gene transfer to the liver and the gonads had decreased.

So, at the 92-day time point, we were seeing about 1 vector copy per 4 cells in the liver, and only about 1 copy per 4,000 cells in the gonads, but only in the highest dosed animals.

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We also did a gonadal distribution study in dogs. In this study, three normal dogs were

injected with AAV null vector at doses ranging from 3.7 to 7 x 1012 vectors genomes per kilogram, and in this study, the vector was delivered using the method that we are using in the clinic. So, a catheter was inserted into the femoral artery and then using fluoroscopic guidance was advanced to the hepatic artery where the vector was infused. Then, semen samples were collected at various times post-injection.

In addition to the semen samples, we also looked at toxicology parameters and also looked at gonadal tissue at the time of sacrifice.

In this experiment, we used the AAV null vector, which contains a promotor list transgene. The reason for using this was just to prevent any CTL response, eliminating the transduced cells.

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So, these are the results of PCR analysis of the dog semen. The lower panel here represents an ethidium bromide stain gel of the PCR products, and over here on the right you can see that the level of sensitivity is about 100 copies per microgram. At this level of sensitivity, there is no evidence of vector sequences in any of the dogs at any of the time points out to day 90.

We also did a southern blot of this gel, and increased the sensitivity down to 10 copies per microgram, which is 1 copy per 30,000 haploid genomes, and again we are not seeing any detection of sequences in the semen of these dogs.

We also performed PCR on gonadal tissue and again we didn't see any evidence of dissemination to the gonads in these animals.

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More recently we have looked at toxicology and biodistribution in the non-human primates, and in this study we have treated 6 cynomolgus monkeys, 2 animals were treated with the excipient, 2 animals got a factor IX vector at a dose of 7 x 1012 into the hepatic artery, and another 2 animals received the same dose of vector via the portal vein.

This study was designed as a toxicology study, but we tried to get some limited biodistribution study by harvesting the liver and the gonads and doing PCR analysis when the animals were sacrificed at day 135

[Slide.

This is the results of that study. What you can is that in 2 of the 4 injected animals, we

saw gene transfer to the liver. It is not really clear why only 2 of the 4 animals worked, but what we can say is that in those 2 animals, gene transfer was relatively efficient, so 1 of the animals that got the vector via hepatic artery, we saw vector genomes at about 1 vector sequence per 3 cells, and in the other animal we saw 1 to 2 vector sequences per cell.

What we also saw was, you know, despite this high level of gene transfer to the liver, we did not detect any sequences in the gonads, and the level of sensitivity in this particular PCR assay was 1 copy per 40,000 diploid cells.

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We also took advantage of this non-human primate testes to ask the question whether any of the cells in the testes had the receptor for AAV, which Jude Samulski's group had previously reported to be heparan sulfate proteoglycan.

So what we are looking at here is a stained section of the non-human private testes, and the heparan sulfate proteoglycan is stained and nuclei are stained blue with DAPI. What you can clearly see is that the receptor, heparan sulfate proteoglycan is present in the basement membranes

surrounding the seminiferous tubules, but none of the spermatogenic cells are staining positive to HSPG, suggesting that these cells would be non-permissive for AAV infection.

What I have just shown you has demonstrated that at least in some animal species, we do see dissemination of AAV vector to gonads, and although we didn't see dissemination of vector to the semen in dogs, Valder Arruda will show some data demonstrating that we do get vector dissemination to semen of rabbits, and Mark Kay also just presented our data from the clinical trial demonstrating that we are seeing vector dissemination in human patients.

So there certainly is the risk for both horizontal and vertical germline transmission. What I would like to present now are some studies that we have been working on and also some published work that addresses the risk of AAV dissemination in both horizontal and vertical transmission.

The first study is a paper from Philip Moray's [ph] lab looking at vector shedding in a number of biological fluids, and then I will present the development of a cell-based infectivity

assay, so that we can now begin to look at biological activity of AAV in semen samples.

Then, I will also address the issue of vertical transmission by describing an experiment that we have initiated in collaboration with Dr. Jon Gordon to see whether AAV can infect murine sperm cells

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So, the study that was published in the Journal of Molecular Therapy last December from Philip Moray's group is shown on this slide. They injected 8 monkeys with an AAV-Epo vector at doses ranging from 5 x 108 to 1 x 1010 infectious units per kilogram, and their vector had a particle infectivity ratio of about 100.

This vector was inject intramuscularly and then at various time points post-injection, a number of body fluids, such as serum, feces, urine, saliva, lacrimal and nasal, but not semen, were evaluated both by PCR for vector sequences and using a replication center assay to look for biologically active AAV.

In addition, they looked in the peripheral blood mononuclear cells for vector sequences

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This is a figure from their paper, which shows the results of the replication center assay. In this assay, cells are coinfected with AAV and the helper virus for AAD, adenovirus. Following incubation for several days, the cells are harvested and filtered onto a nylon membrane and then harbodized to a radioactive probe.

So what we are looking here is the ability of AAV in the presence of its helper virus, to both infect and replicate in this cell.

The panel on the lefthand slide shows the controls. This is AAV that has been spiked just into media, and you can detect 1,000 down to 1 infectious unit. However, when the AAV is spiked into either serum, feces, urine, the level of sensitivity in the assay decreased about 10- to 100-fold.

On the righthand portion of the slide is the results of testing the serum from two of the monkeys, and you can see that 30 minutes post-injection there is evidence of biologically active AAV in the serum, and you can also detect some activity one day and two days post-injection, but by five days post-injection, there is no longer any biological activity in the serum

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This slide just summarizes the results from all 8 monkeys. The red bars indicate a 30-minute time point. The yellow bars represent a one-day time point, and the blue bars represent the two-day time point.

Basically, you can see that in all of the animals, by three to four days post-injection, there is no longer any biologically active AAV in the serum. They also tested other body fluids, but they only found activity in the serum.

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Finally, they also looked for AAV sequences in peripheral blood mononuclear cells, and surprisingly, they were able to detect this signal out to 10 to 15 months post-injection. So vector sequences can be persistently detected in the peripheral blood mononuclear cells

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So, just to summarize their data, AAV vector sequences are detected in all body fluids by PCR for approximately 6 days. I didn't show you this, but they did also demonstrate that the PCR signal is due to packaged AAV sequences rather than free DNA.

They also demonstrated that biologically active AAV was detected in serum for 48 to 72 hours, suggesting that the risk of horizontal transmission is limited to a short period of time post-injection.

Finally, they also concluded that vector sequences can be detected in the PBMCs for as long as 10 to 15 months following an intramuscular administration

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After discussions with the FDA and also following the December RAC meeting, it became clear that it was important to develop an assay, so that we could detect or try to detect biologically active AAV in semen samples, so this just schematically illustrates the assay that we have been developing.

Basically, it is similar to the replication center assay that I just described, however, the readout of replication in this case relies on a quantitative PCR assay rather than a hybridization.

So basically HeLa cells which express the AAR Rep and Cap genes are incubated in the presence of 100 microliters of semen with increasing doses

of an AAV factor IX vector and with adenovirus.

Then, 72 post-injection the cells are harvested, DNA extracted, and subjected to quantitative PCR

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This just depicts a typical result from this assay where the intensity of the red color is meant to represent the amount of PCR amplification detected in the well. So in the case of just spiking vector into media, you can see that we can detect as few as 10 to 50 vector genomes per well, however, when the AAV is spiked into semen, the level of sensitivity of the assay decreases about 10-fold, so that now the lowest dose that results in an amplification signal is 500 vector genomes per well per 100 microliters or 5,000 vector genomes per ml of semen.

So what we intend to do with patient and animal semen is two different assays. First of all, we will simply extract DNA from the sample and do quantitative PCR to determine the number of vector genome per ml, and in addition, we will take a portion of the sample, run the infectivity assay to determine the infectious units per ml of semen, and then we will be able to monitor and compare the

kinetics of clearance of both the physical and the infectious particles.

[Slide.

To address the issue of vertical transmission, as I mentioned, we have initiated a collaboration with Dr. Gordon. In this case, what we propose to do is expose murine sperm cells to very high doses of AAV.

We feel this is a very rigorous test of whether AAV can actually transduce sperm although a very non-natural situation, but this slide just illustrates the steps that are being taken.

First of all, murine sperm are isolated and then exposed to an AAV factor IX vector at an MOI of about 1,000. The sperm are used in an in vitro fertilization, and then the fertilized oocytes are implanted into pseudopregnant females.

The fetuses will be harvested 10 to 12 days post-gestation, DNA will be extracted and subjected to southern blot analysis, and what we will be looking for is single copy AAV factor IX sequences in genomic DNA, and if we are able to detect one copy per diploid genome, that will be used as evidence of vertical germline transmission

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Just to summarize what I have just been discussing, first of all, the extent of vector dissemination to animal tissues correlates with dose and decreases with time.

Following intrahepatic delivery of AAV, vector is either absent from gonadal tissue, which was the case in the dogs and the non-human primates, or present at levels 1,000-fold lower than liver, as in the case with the rats, and in this case it clears with time.

The studies in the non-human primate suggest that AAV in serum is not infectious after 72 hours, but vector signal can be detected in PMBCs for up to 10 months after an intramuscular administration.

The AAV receptor, HSPG, is not expressed on non-human primate spermatogonial cells, suggesting that these cells may not be infected by AAV.

Finally, we believe that the data is consistent with hematogenous dissemination of vector to gonads with clearance over time.

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So the issues that we are continuing to address are, first of all, is there infectious

virus in semen, and as I mentioned, we have developed an infectivity assay which we intend to use both on humans and animal semen samples.

Another question is are the vector sequences in semen associated with the motile sperm, other cells, or the seminal fluid, and as Mark Kay mentioned, we have begun fractionating the human semen samples, and we have also begun doing this with rabbit samples, as you will hear in the next presentation.

Another question that we are trying to answer is can AAV infect mature sperm cells, and we have initiated a study using IVF to demonstrate or not demonstrate this in animal models.

I will stop there.

DR. SALOMON: Thank you very much, Linda.

I was thinking of doing the next talk and then discussing all three of the talks as a group. Would that be okay with everybody? I got the feeling Avigen was kind of packaging this as a group.

Assessing the Risk of Germline Transmission of AAV in a Rabbit Model

Valder Arruda, M.D.

DR. ARRUDA: I would like to talk now

about preclinical studies, address the issue of biodistribution following injection of an AAV vector into rabbits as a model to analyze the inadvertent germline transmission

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Animals in these studies were injected with the same vector to be used in clinical trial. The doses ranged from 1 x 1011 to 1 x 1013 vg/kg.

Semen is collected at serial time points after injections, we intend to fractionate the semen, analyze the total semen and fractions by PCR developed by the human specimens, as Dr. Kay said before.

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Although when we talking collection of semen for rabbits, I like just to mention that the method we are using is the natural method, using an artificial vagina that has an advantage, it provides an uncontaminated sample for each animal.

However, this method has a disadvantage. The animal requires to be trained to do this procedure, and this has some implication, as you will hear later on during my talk.

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When we talk about semen, we talk actually

a marker, what happens in both genital and urinary tract. Actually, 70 percent of what we call ejaculate comes from the seminal vesicle, 20 percent from the prostate gland, and only 5 percent from tests and ducts, and a small portion from accessory glands.

Also, although spermatozoa is the main cellular component of semen, there are other cells that is special for our case is really important to know, and these cells are present normal in fertile donors like leucocytes, epithelial cells, immature germline cells, and enucleated cytoplasm, and this can be around the cells.

Also, for rabbits, are commonly found debris in gel. Gel especially comes from ejaculate of young animals. Together, this explains the reasons why we would like to fractionate the total semen before we save it in aliquot to analyze the total semen, we go for fractionation to obtain the motile sperm and seminal fluid and the normal type

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What we have up to now is actually 3 cohort of animals that has been injected in total 27 rabbits.

The first cohort consists of 12 animals.

They were 5 months old at the time of injection. Although sexually mature, these animals were not experienced in semen collection, so we are restricted to analyze only later time points.

It was necessary to go back and look for experienced animals. At this point, we could get 3 animals. They were 18 months old and semen was collected weekly.

More recently, we have a group of 12 rabbits, median age are around 20 months, and these we obtained from retired breeders.

What I am going to start to show to you is the result of the 10 in the second cohort followed by the third cohort, and only the later time point for the very first group we inject.

[Slide.

For all these animals, serum sample was collected 24 hours injections for the 8 and up to 7 days. For all of them, we have augmented vector sequences by the PCR.

Typically, each sample that has been analyzed for each animal are represented here. We run assay in triplicate with just semen and one spiked experiment to exclude PCR inhibition.

As you can see here, this is the first

experienced rabbits that we inject. At the low dose, no signal was detected, in the triplicate experiment, one single band out of triplicate in the mid-dose cohort, and the higher dosed animal, three out of three. This higher dose, although it is not a qualitative assay, is close to 10 cups of vector plasmid.

[Slide.

So this table shows the serial time points from the three experiments, rabbits ranged for 7 days following injection up to 115 days. Each assay, as you can see here, was run in triplicate in the yellow line, the semen that was detected as a positive signal. For the lower dose animal, we never detected any signal during this period. For the mid-dose animal, signal has been detected up to day 22, for the higher dose, up to day 44

[Slide.

We attempt to fractionate the rabbit semen and the optimal fractionation actually depend on the size and shape of the sperm, as well as the pH of the semen. At the very first time point, we use parameters worked out for human semen, and actually reagents for human semen, and when you look under the microscope, we saw a lot of agglutinations,

cell debris. You can see that even after fall in fractionation, you concentrate fraction of motile sperm, but it still has a lot of debris.

[Slide.

When one compares germ cells for human and rabbits, they are different, so the volume of ejaculate is smaller in rabbits, and we anticipate that this would be a problem for fractionation as for humans, although the density of the sperm in rabbits is higher, the characteristics of this sperm is different. They are pretty much the same total length, but the distribution is different.

[Slide.

So, we talked with people at this company, Nidacon, and they actually in-house some reagents to use for rabbits. We didn't use if that was really helpful or not, so we just took a chance and we used the reagents that have been developed for rabbits. Not only the grade had changed, but also the centrifugation conditions changed.

After that, we improved the fractionation, but occasionally, we still detect 1 or 2 percent of cells other than motile sperm.

[Slide.

So these results are from the first three

experienced animals. The top animal is what I showed before. So these are the points that we are able to fractionate the semen in these animals.

As you can see, the motile sperm analysis shows a positive signal in the mid-dose animal. The high-dose animal, at this point, the volume was not enough to allow fractionation. It was just 200 microliters. So, we saved it only for the total semen analysis.

After day 7, the second time point was day 22, and all the animals turns out to be negative, and up to here, we use the human protocol, and after this, the rabbit protocol, but after that, as you can see, no signal has been identified by the same PCR reaction.

For the normal type sperm, seminal fluids, again, we have seen signal positive for the mid-dose group and from the higher dose group, and again, for low-dose animal, we have never been able to detect

[Slide.

Now, I will show the third cohort. these are 12 rabbits, experience rabbits, and we have only two time points. It is important here that we have time point 7 - 15 days, 15 days we didn't have

for the very first group, we just skipped to day 22nd.

This is the total semen. You can see that three animals on the low dose cohort was positive at 7 days, but became negative at 15 days. For the mid-dose and the high-dose, these animals are still positive although decrease in numbers at the normal type sperm fraction, also we can see that the higher the dose, the higher the number of animals positive up to this early time point.

The motile sperm analysis, we have not observed any positive signal for the lower dose animal, a positive signal for the mid-dose and high-dose, and again I would say that at this point, the positive didn't change much from 7 to 15 days. We still collect today, actually, the day 21.

The last group, these are the first cohort that we inject that we inexperienced at that time, so it took us like a couple weeks to train these animals and now they are able to provide the specimen.

So we have here, we collect semen for groups that were injected a week apart. That is why we have this range of days, from the low, mid,

and high dose, they are persistent negatively until day 132.

It is interesting as Dr. Couto showed before, in non-human primates, one can detect peripheral blood mononuclear blood cells positive at late time points following AAV injection. Here, we also have been able to detect that these animals, they present positive signal in their peripheral white blood cells, and the top panel shows, at the same time, which corresponds to three months following injection, the total semen are negative.

I am not going to go into detail into the rabbit experiment, this is just to represent a schematic, a very simplistic overview to say that these are numbers of days that get usually a spermatogen cycle in rabbits takes up to 42 days.

Initially, the stem cell, it is outside the protected area, so outside the blood-testis barrier. After day 16, cross the blood barrier, came to spermatocytes, and takes up to 10 days from the mature cells, spermatozoa, to get to the semen.

So, you assume that the stem cell has been exposed to a vector at day zero. The first time point that one animal should show up a positive

signal in the semen started at day 52, maybe with a peak at day 58, and after that, you have the steady-state signal.

If you put back the three cohorts of animals we inject so far, we can tell the following. We detect PCR-positive signal in total semen or some fractions, 7, 15, 20, and 44 days. The PCR becomes negative, the old sample tests after day 50. This is for the first three cohorts of animals, and this is for the very first 12 animals that we start collecting at day 86 up to day 1 to 132.

If we consider that the rabbits spermatogenesis single sites 44, 42, 46 days, at this time point that we analyze, we will be able to analyze at least two to three sites of the total rabbit spermatogenesis.

Although it doesn't look like we are transducing any immature or stem cell at this point following only two or three sites of spermatogenesis, there has been talk before here was that possibility that actually the vector cross the basal compartment, cross the blood-testis barrier, and gets into more mature cells at this point.

I even should skip this, but I will try to do what has been said before that may not work, and didn't work, that is was put multi-sperm of these rabbits in culture. This is just to show -- I hope you can appreciate these are spermatozoas, and these we still found some cells into the motile sperm fraction, and unfortunately, by this computer thing, we cannot make the picture come out.

What we did, we exposed these cells to AAV2 under a CMV control expressing a GFP. The MOI used a range from 1 to 5,000, and the committee has a cut that we provide, shows that only that cell that I identify here, it looks like a bean or something like that, actually turns out to be positive for GFP. Any other, the motile cells were positive.

So, initially, for the muscle trial, we performed pretty much a similar series of experiments, used the same model, the rabbit, and I would just like to summarize this. This actually has been published in 2001, and what we are able to identify following intramuscular injection of AAV2 into rabbits was the following.

We performed a series of IF staining and FISH analysis shows that we can detect signal from

the vector, and this is localized in the vessel wall and the testicular basement membrane, which are rich structures for heparan sulfate proteoglycan, which there is no receptor for AAV serotype 2.

The detectable signal especially in the gonads disappears with time. It is important to remember that in this cohort of rabbits, no semen signal was ever identified, and also into the gonads, we neither detected any intracellular signal when you analyze animals following 7, 36, and 90 days.

This is just to represent what we believe that the signal, this is IF staining, what you call localization for the AAV capsid and for heparan sulfate proteoglycan on the vessel wall in the testis basement membrane.

So, in the last experiment that I would like to show is attempt to transduce not mature spermatozoa, but immature spermatozoa. In this we have murine cells in culture in which murine spermatogonia and Sertoli cells were co-cultured.

We transduced again with AAV2 under control of the CMV promotor expressing lacZ at the MOI 5000, and we stain for x-gal. Here, it showed

the signal. Just before, I should say, that we identified the spermatogonia by immunostaining with a monoclonal antibodies to germ cell nuclei antigen.

This is the result. At the bottom is the mouse spermatogonia and Sertoli cells that give this kind of reddish signal. In contrast, if you take the same transduced model, the fibroblasts or human [inaudible] it turns blue.

I would like to conclude that intravenous administration of the dose of AAV up to 1 x 1013 in rabbits results in transient detectable signal in semen in a dose-dependent manner.

PRC positivity of the semen persists up to day 44 in that cohort, that we have follow-up for almost 100 days revealed no positive signal, which is a duration of 2 or 3 times of the rabbit spermatogenesis.

Vector signal can be detected in peripheral mononuclear blood cells for at least three months in rabbits in contrast to non-human primates, we know that this can go up to 10 months, but the vector is not biologically active after day 7.

In ongoing experiments, you can predict

that we will continue follow up with kinetic clearance, determination of anatomic localization of signal as a function of the vector dose following intravascular injection.

To determine whether AAV infectivity is detected in rabbit semen, we are followed by the experiments that Dr. Couto has before. As well, we would like to determine whether receptor for AAV2 is present in mice, rabbit, and human spermatozoa. That could give us some idea.

I will stop here.

DR. SALOMON: I want to make sure we all understand where we are going. What we are going to do now is have a discussion of the three talks that came, followed by people who have been invited to speak in the public hearing.

We had talked about moving that out of order, but I am told that is not proper, but to reassure everybody that we will discuss the questions to the committee after that, so that everything will be on the table before we get to the questions.

I think there are a number of interesting issues raised by the these three presentations, and I would like to put those open for some discussion.

Dr. Dym.

Q&A

DR. DYM: I had a question for Dr. Couto, please, just a clarification, and maybe I didn't understand some things, but when you showed very elegantly that the receptor for AAV is around the seminiferous tubule and in the interstitial spaces, not inside the tubule, but then you didn't show AAV in the gonads.

Isn't there a discrepancy there? Shouldn't it show up in gonad if the receptor is there, or did I miss something?

DR. COUTO: That particular section was an animal that was not even injected with an AAV vector, so we were just strictly looking at to tell whether the receptor for AAV is even present in a non-human primate testis.

DR. DYM: But in your other monkey tissue, didn't you say it is not detectable in testis?

DR. COUTO: The AAV sequences are not detectable in the gonadal tissue, correct, by PCR.

DR. DYM: So, wouldn't they be there, because the receptor is there? I am missing something.

DR. HIGH: Can I clarify that question?

So, the answer to your very perceptive question, if you look in the Molecular Therapy paper, there is actually a FISH analysis of a rabbit testis from an animal sacrificed at day 7, after injection with AAV, and in that, you can see tracking in the same location that Dr. Couto showed in the non-human primate testis.

You can see AAV vector detected by FISH analysis in the same location along the testicular basement membrane and actually around the vessel wall, as well. You can detect that at day 7, but by longer time points, which were presented in several of the studies that Dr. Couto did, both in rabbits and in other species, as well, if you look at later time points like 50 days after or 100 days or 135 days, you don't see AAV vector any longer in the gonadal tissue.

So, your point is correct, and if you look early on, you can see that, and that has been published in that Molecular Therapy study or day 7, but at later time points you don't see it.

DR. SALOMON: I have a couple questions. Going back to the very beginning, I posed a question about if sperm were not transcriptionally active, then, how do you interpret an experiment

where you put in a CMV-GFP vector?

DR. ARRUDA: I don't think that is the ideal experiment. We just want to see if one could transduce motile sperm in culture. There are some people that say they can, in fact, transduce some mature spermatozoa using more aggressive ways. We do not expect anything else. It wasn't a surprise that the results were negative, but I think the best way to answer your question is to perform an experiment that Dr. Couto is doing with transducer cells in culture, and then you do in vitro fertilization and see the outcome.

DR. SALOMON: I certainly don't think that the evidence that you didn't get GFP expression really addressed anything.

DR. ARRUDA: I agree.

DR. SALOMON: If it had been positive, I agree it would have been important, but I don't know what is the point in showing it as negative.

DR. ARRUDA: I agree with that, and also it has been published that one can detect lacZ in this spermatozoa.

DR. SALOMON: What I would like to hear some discussion of is whether the experiment that Dr. Couto presented, and I guess that is going to

be done with you, Jon, is the best study.

I have some concerns about that. It is a very good study. It seems to me that it is really almost going over the top, which you said yourself, Jon, was what you should do. So I would like to hear your comment on that as a point of discussion.

It seems to me you have a reagent, however, that would also be extremely useful, and that would be to take your AAV-CMV promoter GFP and put it into the rabbit, and then actually trace GFP expression in different compartments particularly in this case, of course, in the spermatogonia, I mean so you could do it at different -- I don't need to tell you all the different variations of that, but that seems to me to be the most physiologic experiment.

DR. GORDON: I want to make a brief comment on that. It is no surprise that sperm will not express genes put into them, but that doesn't mean that genes aren't in there, and couldn't be expressed late, just to reemphasize the strategy of doing this IVF.

The other comment i would make is if there were AAV-CMV lacZ, I haven't looked for GFP in embryos, although I am sure it can be done, but if

there were lacZ vectors, where I know I can look, you could look at thousands of cells in a very short time after exposing sperm to such a vector, and just simply stainings on an intact embryo. We did that for adenovirus, it worked really well, and that would be a very nice protection against contamination when one harvests fetuses and the strategy that we are taking, which is of major concern to us, and which has been discussed amongst us over the last few weeks.

DR. COUTO: There is another experiment that we have thought of doing, and it is based on an experiment that Bob Braun's lab has done with adenovirus, where they had an adenovirus that had an expression cassette that has a protamine promoter hooked up to a lacZ vector.

In that case, they are doing a natural route of administration, and then looking to see whether all of the progenitor spermatogonia, spermatocytes, et cetera, in an entire seminiferous tubule turn blue over the course of time. That is another experiment that would not only look at the mature sperm, but also the immature sperm.

DR. SALOMON: The idea there is to use the protamine promoter as kind of a tissue-specific

promoter, so that is even cooler, as would say in California.

Jon, the question that I had for you was the experiment that they are talking about where you essentially culture the sperm with 1,000 MOI and then you go and do IBF. Isn't that just going to have a bunch of DNA coating the sperm? Don't we already know the results of this experiment before they do it?

DR. GORDON: Meaning you think it would be positive. Well, I can just say that we did that with adenovirus. The rationale of this -- and it was not positive -- the rationale for this is that if AAV arrives to semen, then, it can expose motile sperm, and in the rabbit, motile sperm seemed to be associated with AAV.

So the question then is can these carry the genomes into the embryo via the natural fertilization process. As I pointed out in my talk, that is not easy to do, and we certainly did not find that to be the case with adenovirus, even at 100 viruses per cell, so we would not predict it to be positive, mostly because of the investments of the sperm are mostly lost en route through the zona, and so on.

So we don't expect it to be positive. If it's positive, we have to look at why that is true, whether it is really transduction or whether it is so much AAV in our IBF prep that we couldn't get rid of it all, something like that.

DR. SALOMON: So, Jon, I have to ask the stupid question. So why did your DNA experiments work when you coated the sperm, so why does that work?

DR. GORDON: That works only if you load the sperm into a microneedle, push the microneedle through the zona, then through the plasma membrane of the egg, and then insert the sperm with the DNA around it directly into the cytoplasm. That works. I say to you that I don't believe the Cell paper which said that just mixing it with DNA and doing IBF works, since no one seems to be able to repeat it including me.

DR. SALOMON: Thank you for that clarification.

DR. KAY: I just want to make a comment that even under the very worse scenario, where you do get carrying in of the single-stranded genome into the embryo, at very low copy number, even one copy, the chances that that single-stranded genome

is going to become double-stranded is very low.

DR. GORDON: Another final comment about the thing I said about exposing them and then looking for expression in embryos, that is a problem with the kinetics of AAV genome activation. It takes a long time, as I understand it, to actually turn the genes on, and so the experiment is a little bit less easy to do with that, as are experiments with protamine promoters, and so on, with AAV, just because it takes a long time to turn the genome on.

DR. SALOMON: Dr. Rao.

DR. RAO: This is for Dr. Arruda. There are two things which weren't absolutely clear to me in your presentation. When you showed the spermatogonia in culture and you looked at AAV infection with the high MOI of infection, there were some infection. You showed 293's at the same time?

DR. ARRUDA: Yes. We have two cell lines as control. Those are 293 cells with human cell line and the murine cell line, the fibroblasts, that was positive.

DR. RAO: But the spermatogonia were not?

DR. ARRUDA: The murine spermatogonia was

not. That experiment was murine spermatogonia.

DR. RAO: And when you see positivity in the rabbit motile sperm, fractionated sperm, where do you think the virus is there? I mean you fractionate the rabbit sperm.

DR. ARRUDA: Yes.

DR. RAO: And you take the motile fraction, which you now have purified.

DR. ARRUDA: Yes.

DR. RAO: You think there are no contaminating cells, right? And you see by PCR that there is some positivity, right?

DR. ARRUDA: That's correct.

DR. RAO: Where do you think that is coming from?

DR. ARRUDA: If you remember, even when we use what we call the optimal protocol, it is a list developed for rabbits, reagents for rabbits, we still see some debris, which you don't know which kind of cells are those, and also no motile sperm cells, other than, any other, so it is a more concentrated fraction of motile sperm up to 87 or 95 percent, but we still see some of those.

Just from technical reasons like if the animal has very little, urinated during the

procedure, you are contaminated, you don't see that unless the thing turns out to be yellowish, things like this.

DR. SALOMON: Dr. High.

DR. HIGH: I just want to underscore the point that you raised in that question, because it is really critical to the kind of analysis that we are trying to do, and I am not sure we are really on the right track.

That is, that what semen fractionation does is enrich for motile sperm, but it doesn't really exclude all other cell types, so when you take semen fractionation and couple it with a procedure like PCR, which will certainly detect small amounts of contaminating material that may not be from motile sperm, then, have we really come up with the best test. So your point is well made.

DR. DYM: This is for Dr. Kay. It is sort of a comment, maybe a question related to maybe why it seems difficult to get the vectors into the germline or into the gonads.

When you put it into the liver, it, of course, goes straight into the liver, maybe a little bit goes via the artery, gastroduodenal artery to the upper part of the GI tract, and then

it goes into the capillary spaces, and so on, then back to the heart, back to the aorta.

Now, those testicular arteries, I know them very well in the human also. They are tiny, little things, and it may not just get down there again.

DR. KAY: I actually would say that it probably does, and let me explain how the liver flow works. First of all, the catheter is ballooned at a point that is past the gastroduodenal, so there shouldn't be backflow into that artery directly.

However, even by clamping off the hepatic artery, most of the blood flow through the liver still occurs because 60 percent of blood flow through the liver is through the portal vasculature.

So, what we suspect happens is that you get actually infusion into the liver, and that you actually get washing into the venous side through the portal circulation into the vena cava, and then you get disseminated flow.

If you look at animals in biodistribution studies that have got an hepatic artery or portal vein, or what have you, you do find the vector in

other tissues, but at very low concentrations. To the best that we can tell, at a reasonable rate, and, you know, we can define that statistically or not, the only other tissue that we have really seen anything that would be even suggestive of transduced cells is a rare positive cell in the spleen.

DR. DYM: Do you know then the clearance of this?

DR. KAY: No, we don't know that. That is something that we are actually working on in animal models. It is almost impossible to do in mice, and we have developed some surgical techniques in the rat where we have actually clamped off the vena cava in the portal vein and then just infusing the liver, let it sit at different dwell times and then release the clamp, and then are looking at how much gets into the rest of the circulation.

I think the question is how much of it gets into the liver on the first pass, and things like that, and we don't have a definitive answer for that, but we are doing studies as best we can to try to address it.

DR. SALOMON: Dr. Gordon.

DR. GORDON: I just wanted to reemphasize

again this discussion with Dr. High that just preceded that, if the motile sperm fractionation results in the detection of this material, that doesn't mean that it is on the sperm, but it certainly means that the sperm could come in contact with it. That is really the issue in these IBF strategies.

If the sperm can come in contact with it, does that mean that they can carry it in and cause vertical germline transmission, and that is why I feel it is necessary to make sure that you do that experiment.

I can tell you that with adenovirus they don't. I think it is hard to do that, and I don't think it will happen with AAV either, but a discussion of exactly where it is sitting in the motile sperm fraction isn't really that relevant. You know that that means it could come in contact with motile sperm.

DR. SALOMON: Do we know that there is any heparan sulfate proteoglycans on the sperm itself? I know that they showed the picture that the spermatogonia and the seminiferous tubules seemed to be negative.

DR. GORDON: I don't know the answer to

that.

DR. SALOMON: I guess that follows up to me a question. That is, you showed the HSG expression in the seminiferous tubule as a point like reassuring us that the target wasn't there. I am not an expert on AAV, so I defer to my colleagues on this.

I mean is that it? I mean there is not other cell attachment molecule? I mean if so, that would seem to be rather unique since every time I think that is it, there is always something else.

Jude? Obviously, I am looking at you.

DR. SAMULSKI: It has been shown that FGF will also bind to virus, and we know that alpha-V, beta 5 is like a co-receptor, so it is not an all or none scenario, but it is a good indicator if those cells are likely to take virus up. You typically heparan. It is not just heparan itself either, it's high sulfonated heparan, so there is different kinds of heparan.

DR. MULLIGAN: The infection point in the rabbit, how does the rabbit compare for AAV2 relative to other kinds of cells? That is obviously, the model is only as good as how sensitive it is. Is there a way you have tried to

look at a range of different rabbit kinds of cells versus other kinds of cells, human cells to see if rabbit cells are equally, in a general way, susceptible to infection? There is definitely in the AAV serotype business, great differences, not only species differences, but also obviously, tissue differences?

DR. ARRUDA: We have some idea. As I said, we inject the same vector that has been used in the clinical trials, so it is expressing human factor IX, and the cohort of animals, that is of the highest dose, we are able to detect human factor IX in the rabbit plasma.

So what this tells us is that I can say how it transduce efficiently in liver cells.

DR. MULLIGAN: Comparable to the amount you have?

DR. ARRUDA: No, it's lower than because the major difference is that we did I.V. infusion, not deliver into the hepatic artery, and if you do these in the same animal, you see 5 or even less expression follow I.V. That is why we have to go into the hepatic artery.

DR. MULLIGAN: If you just compare I.V. and the amounts versus the rabbit, is it

comparable?

DR. ARRUDA: I would say not, because the mouse, we inject -- you can correct me -- some of this, like C57, they respond very well to I.V. infusion compared with even other strains.

DR. KAY: I think there is a complicating factor and that using human factor IX in a rabbit, and do the rabbits develop inhibitors? I mean because you get a slow rise of expression over time, you may never hit the peak level, and in mice, you know, it is very dependent on different strains.

DR. MULLIGAN: I know it's complicated, but I mean that ultimately the question is whether or not there is a way to have a sense of whether or not the rabbit is as susceptible. I mean the argument in a way goes to your favor in that the animal models for AAV may not be that good because of the differences, like with the VSVGs, a pseudotype, you can infect all kinds of different, 1,000 tissues.

AAV seems somewhat different as a vector system because there are such big differences from species to species and tissue and tissue. So it is not clear whether the rabbit would be a better or

worse system, but it would be nice to have a sense of typical tissues that people attempt to do transduction with, is it comparable.

DR. SALOMON: So you want to see data in a rabbit, for example, showing that intrahepatic artery injection has a somewhat similar transduction efficiency.

DR. MULLIGAN: LacZ and muscle, in a rabbit muscle, looking at the number of positive muscles -- Jude, you must have done this sort of thing. No?

DR. SAMULSKI: Officially, we have not done this experiment.

DR. MULLIGAN: How about unofficially?

DR. SAMULSKI: Unofficially, we haven't done it either.

[Laughter.]

DR. KAY: Can I raise the issue of hematogenous spread again? I mean this vector, unlike the retroviruses and other vectors that are being used, that have a potential to integrate, are not pseudotyped, and they basically represent the capsid of the wild-type virus.

During wild-type infection, there is going to be some hematogenous spread although I am not

sure that anyone knows what the concentration is, and yet we haven't had any evidence of the AAV sequences into the human germline, unlike what has happened with most mammals with retroviruses.

So, I think, in nature, that there is some hematogenous spread of the wild-type virus, yet it hasn't been detected in our germline.

Any comment on that?

DR. MULLIGAN: I want to switch back to this infection question. In the case of doing in vitro infections, there are different kinds of cells, my impression was that people often with AAV use very, very high multiplicities of infection like 10,000 to 1, or 50,000 to 1.

Do you feel comfortable that you have really, in the in vitro rabbit infections, really dosed, put on a virus to potentially detect something?

The question was, when you do in vitro infections, different kinds of cells, what is the maximum multiplicity of infection that you use to see if something could be infected? Is it 5,000 or is it 50,000?

DR. SAMULSKI: In our hands, we have seen things like certain fibroblasts or real refractory,

and you need about 100,000 particles to it transduced.

DR. MULLIGAN: That was my impression. So the question is, have you really, with all the vagaries of the system, have you really given it the greater shot, unless I got that wrong, you did 5,000 was your multiplicity of infection? Was there any reason you didn't test 10 times that? No.

DR. SALOMON: Trying to get some sort of themes going here, it seems to me at least three different things could be discussed. The first would be the idea that the adenovirus associates with the sperm or with -- well, actually with the sperm itself, and therefore, would be carried into the female and might then enter the egg at the time of the sperm's fusion, and inadvertently deliver the genetic material from the virus. That's one possibility.

I think that that possibility, Dr. Gordon's and your experiment would address, so that is a good thing, we don't have any data yet, but it sounds like you have got that on track.

The second possibility would be that the sperm are carrying the virus, and I am not

convinced that any of the tests we have seen so far adequately address that. I am not trying to be overcritical either, because I can see how difficult some of these studies are to do, and commend you for doing things like figuring out how to fractionate rabbit sperm, and it shows how careful you are trying to be.

But it still seems to me that when you are dealing with literally millions of sperm in a typical ejaculate, and you are doing PCR studies that were sensitive down to 1 in 30,000, that this is not going to work, I mean that that is not very convincing, and specific studies of looking at in vivo expression, or whether you use the GMP, the CMD promoter or the protamine promoter, something along those lines haven't been done yet.

I don't think that we really know the answer to that part. I guess a third thing that occurs to me is that regardless of the germline transfer question, if semen of male patients had got the vector for weeks and weeks, your patient at the lowest dose is 14 weeks positive in semen, is that going to get transferred to vaginal cells and other cells in mothers?

We all know through bitter experience with

HIV that that is a portal to the blood, as well. You know, how likely is it even if we just focus on the semen positivity, to which there is no conflict, right, I mean we all agree there is semen positivity, are we going to see a lot of the partners infected and how does that impact on issues in terms of doing these studies?

DR. COUTO: One thing that we are trying to do to address that is with the development of this infectivity assay, at least try to demonstrate that there possibly is, even if it's there, it's not infectious.

There may be vector sequences there in the semen, but after a couple of days, maybe it's no longer infectious, so that is one thing that would address that.

DR. SALOMON: So your assay would -- infectious, though -- you are saying won't replicate if you add helper virus and wild-type adeno for the Rep and Cap genes, but it doesn't really address whether it just delivers the payload gene, right? How likely will it be? Tell me if I am being dumb.

But I mean how likely would it be that a positive semen that actually has adenoviral capsid,

that you inject it earlier in the hepatic artery, will, when injected during sexual intercourse into the female, just transfer it to vagina mucosa?

DR. COUTO: If it is able to infect the cells and you provide adenoviral help, it should be able to replicate, and that is really what we are asking in that assay, so I think we can rule that out.

DR. KAY: The Mollier [ph] data suggested that at least in the high end injected in the blood, although the vector DNA was detected for a long period of time, the biological activity of those particles diminished to undetectable levels after a very short time.

So what I think Linda is trying to say, just to reiterate, is that there is a reasonable chance that the DNA or the particle could stick around, but it may not be actually infectious or able to transduce a cell.

If it went into like, say, a vaginal epithelial cell one time, you would never see that. I mean it would be a single transduction event, it wouldn't replicate. I guess the question is if it was carried in on the sperm into an oocyte, what is the chance of naked DNA or DNA that is partially

exposed could get through the zona pellucida during a fertilization event.

DR. GORDON: All I can say to that is I don't think it has ever been seen in the literature despite claims to the contrary.

DR. SALOMON: I wasn't trying to make things too complex. I guess I was just saying that we are really not discussing just the Avigen factor IX study even though that is on the table here, my feeling here is we are discussing just in general issues here. I greatly respect Avigen being here and presenting it, because it is always great to have a specific study to focus on, but we also don't want to lose sight of the fact that there are bigger issues here.

So I am saying that a lot of different clinical trials could come along following potential success in the Avigen trial, and I certainly do wish you the best with this one. Those could deliver gene payloads that could be a lot more serious than delivering some extra factor IX to a woman inadvertently, so that is really all I am trying to say is if you start delivering -- oh, who knows, I don't want to make stuff up -- but, you know, just a gene payload that might be

toxic, whether that would be delivered to the vagina of the woman and produce some problem there.

Again, I am not trying to make that a big killer issue, but it seems like from everything I have heard that it is still theoretically possible.

DR. MULLIGAN: One thing that Jon's -- what do you call them, provocative experiment -- would test is, in principle, whether or not any AAV, since that is the worst case, we are soaking things with the AAV, and then you are doing the most efficient means of sex, maybe not the most efficient, but you are doing it so you are opening up as much AAV as possible, so you could look for things in addition to the integrated sequences.

I think I saw that you were going to test only for integrated sequences. After the in vitro fertilization experiment, it might be useful to, since you have the material, to look for whether or not there is AAV. Presumably, if it wasn't integrated, it would be dramatically diluted, but it would be interesting to see if you could detect it, because that would address in a sense the worst case of whether or not, during sexual intercourse, you can transfer AAV, and it can persist maybe as an unintegrated form, but these is some infection.

DR. GORDON: We are doing that.

DR. SAMULSKI: I have a question, and it is more a curiosity. It seems that the so-called debris and other contaminants are a major contributor to the positive results, and I am wondering if people here have felt that the efforts to purify these different fractions have been exhaustively done, because it seems that when you move to other reagents, whether they are oligos or plasmids, this is going to come up over and over again.

If there is more energy put into the first step of the assay, of collecting and fractionating, will we move away from these long risk things and get into a better assay that is going to tell us there is something worth paying attention to. Again, I turn it back this way, because when you hear someone say this was optimized for rabbits, and this was optimized for humans, does that mean it has been done for 20 years and optimized, or is just gives them the result they need to get something away from something.

DR. GORDON: I just want to very briefly comment on that. Even a fraction of motile sperm is a very heterogeneous population of cells. I

mean some of them have two heads. Some of them have a huge cytoplasmic droplet, which can be close to the volume of a sperm.

So when you actually try to do an absolutely totally pure separation of motile sperm from everything else with a similar density, similar parameters of measurement, similar configuration, it is very, very difficult, and I think that if you try to solve this problem that way, by getting a golden fractionation procedure, you are going to be chasing your tail for a long time, not that I want to introduce other tails into the discussion.

DR. JUENGST: So, thinking generically, kind of at the policy level, I think I learned two things this morning that increased my sense that there are risks here. The first one was the idea that it is not just the integration of a factor IX gene in a harmless place on chromosome 19, but the random integration of genes from the episome presence of the vector.

The second was the increased risk even if through natural fertilization, it looks low with artificial means of fertilization, ICSI and infertility techniques, so it looks like the

patients who are at greatest risk, hypothetically speaking, worst case scenario, would be gene therapy patients who then had fertility problems and needed to go to a fertility clinic.

DR. SALOMON: To Jude's question, the other way around here would be there is still no evidence that these vectors are getting into the spermatogonia, so if you could do enough really well designed, basic, preclinical work, you might be able to make a good case that you just monitor the semen, and not be obsessing about all this purification, et cetera, you know, if you could convince yourself that it wasn't specifically being carried in the germline package of the sperm.

DR. GORDON: Let me just say that I think that is a very important point because if you cannot transduce the spermatogonia, then, when the semen are clear, you can feel that they will be clear, and that not another wave of spermatogenesis will provide more positive sperm to the ejaculate.

DR. SALOMON: Certainly, the data presented today still do not give us any cause -- you know, there is no smoking gun yet that these are being delivered to germline cells.

DR. MULLIGAN: Jon's work wouldn't address

the worst case for trying to get an earlier precursor infected, right? I mean that is, you could think of the same Jon kind of approach where you would put in as much AAV into exactly right time and location, to do the same sort of worst case, and that probably would be the ultimate worst case.

DR. SALOMON: Yes. In fact, that was the point I was making, too, earlier. There was the issue of whether it got in or didn't, but his experiment addressed the latter, right, where it was just attached to the outside.

But the experiments haven't been done yet or designed yet or proposed yet to do the ones that we both suggested, and that is, prove yes or no, whether it gets into the spermatogonia, and if you could get out of that, then, you could make the FDA and the sponsor's life a lot easier.

DR. GORDON: Well, I just want to say again that we have an abstract today at ASGT, in which we are developing this technique of perfusing intact seminiferous tubules with very high concentrations of vector. I showed some of the stuff from adeno expressing lacZ, and again that would be a very highly provocative test.

It doesn't seem to disturb the spermatogenesis much, if at all, and that, with nucleic acid hybridizations, you wouldn't have to rely on promoters and vectors with delayed expressions, which is AAV, would I think be a good standard to arrive to.

DR. NOGUCHI: Just to follow up a little bit on how provocative you be, Jon, wouldn't the most and even more provocative state be to expose sperm to AAV, and then immediately do ICSI, and then look at the outcomes of that?

DR. GORDON: I think that would work because then all the natural barriers to getting it in would be circumvented, but I do emphasize those are natural barriers and that is an artifactual situation, however, as I was saying before in my talk, my official talk, I mean there is a lot of clinical activity where these barriers are bypassed, and I think that we should begin to be interested in that subject, and I don't think that is the subject for this meeting, but I think it is a subject that the FDA needs to begin to get interested in.

DR. MULLIGAN: I like Phil's approach because then it is really more directed an

integration question. It is like doing a transgenic system where you dump in more and more AAV in exactly the right -- for something that happened, and you can see whether it does or doesn't happen.

DR. NOGUCHI: It actually pertains in a way, based on the discussion here, to this experiment, as well, if, in fact, you have the presence of vector even if it's not integrated, but it is around, it could coat the sperm or it could be attached to the sperm. That is the equivalent of what we are talking about. You have a vector, a sperm, a union with an egg, and things.

So I think they are two different things. One is, is there integration into the actual person being treated, and then the other part, can there be a transmission by other than biological means, but just by pure mechanical. That is an issue that pertains, and it is related also to the question of how much sensitivity is enough if we are going to be talking about barrier contraception as a means to mitigate this period of washout, you know, how much washout is enough.

DR. SALOMON: I guess as long as we are at the most provocative experiment discussion, I mean

you could simply inject the AAV into the egg, and then with the lacZ, and ask then in like a mouse embryo or in a chicken embryo or in a rabbit embryo, where it was distributed, and ask the question whether there is some unusual integration or whether it just quickly segregated.

DR. GORDON: Just a brief comment on that. We have another abstract at ASGT, about a adenovirus injection directly into embryo, and what we were doing was asking the question, does this intricate cycle of virus on coating and translocation of the genome to the nucleus, are these obligate steps for expression, which has always been assumed, but never been proven.

Now, the one-celled embryo appears not to have a receptor for adenovirus from our experiments, and so what we did was injected the virus directly into the cytoplasm and said, well, we will bypass the endosome and see what we can get.

We never lacZ expression under those circumstances. We then say, well, let's help the virus even more, we will put it right into the pronucleus, and we have done that. In that case, we see low rates of embryos that express which

appear consistent with perhaps viral genomes that have been partially shredded by freezing and thawing.

To confirm that, we took the viral genome and just injected the pure DNA and got a very similar result. So in adeno, it doesn't appear that the virus can actually do its thing if it's not allowed to go through the regular cycle of infection, but AAV, which is why I asked about uncoating, when it uncoats, if you put that directly in the pronucleus, it is a single-stranded genome, but there is very active repair mechanisms in the pronucleus, and all of that, I should think it would work, but I don't think it has ever been tried. I would be happy to try it if someone wants me to try it.

DR. SAMULSKI: So, we actually did those experiments, and if you remove the zona pellucida and inject the virus, you can get blastocysts to turn blue, so it will transduce those cells. It doesn't work with adenovirus, just like you said, for the same reason. When it comes out of endosomes, there is a proteolytic cleavage that is responsible for the virus on coat, and if you don't go through that pathway, it won't go through.

DR. KAY: Is it integrated?

DR. SAMULSKI: We only did in vitro and carried them out and were able to show that it would transduce those cells, and when we started to collaborate with our colleagues to implant them back in for embryos, the postdoc left, and all of this stuff stopped, so we didn't do any more on it.

DR. GORDON: What was the helper for that?

DR. SAMULSKI: There was no helper. We were simply trying to find a better way of making transgenic animals using AAV as a way of delivering genes, and showed that once could physically put it in, it would transduce those cells, so I think Phil's question is partially answered, you will get it in, and it will work by some mechanical mean.

And far as the stability, integration, all of that stuff, there are no answers at all. It was just lacZ.

DR. MULLIGAN: What was the number of virus particles?

DR. SAMULSKI: It was extremely high. We were putting in about 1010. For a number of reasons, it partially was -- well, we could talk about it later.

DR. SALOMON: Any other discussions here?

What the committee should feel at this point is that just in terms of science presented, that we are comfortable with the main issues, and then we will do the public comment and go on to answering the specific questions from the FDA.

Open Public Hearing

DR. SALOMON: What I would like to do is introduce the public comment. Five minutes have been allotted to each of three speakers.

The first speaker that I would like to invite up is Mr. Steven Humes of the National Hemophilia Foundation.

MR. HUMES: Good afternoon. My name is Steven Humes and I am the Director of Research at the National Hemophilia Foundation, hereinafter referred to as NHF.

NHF is a not-for-profit organization dedicated to improving the quality of life for all individuals with hemophilia and other bleeding disorders. Today, we thank the members of the Biological Response Modifiers Advisory Committee for allowing us the opportunity to provide testimony on recent reports of the presence of adeno-associated virus, or AAV, in the seminal fluid of individuals participating in a hemophilia

factor IX liver-directed gene transfer trial.

There are at least 10 proteins in our blood, which must work in a precise sequence to make the blood clot. A deficiency in any one of these proteins can lead to abnormal bleeding. Hemophilia A is caused by a deficiency of or defect in a clotting protein known as factor VIII. A deficiency of or defect in clotting factor IX causes hemophilia B. Both forms of hemophilia and other bleeding disorders are X-chromosome linked recessive genetic disorders. In the United States, there are approximately 17,000 individuals living with a diagnosis of hemophilia.

Hemophilia manifests itself by easy bruisability and recurrent bleeding into joints and muscles as well as bleeding intra-abdominally and into the central nervous system. The severity of an individual's hemophilia is determined by the amount of circulating clotting factor. The majority of individuals affected with hemophilia have severe disease.

Individuals with severe hemophilia typically have eight to 10 bleeding episodes each month. This chronically recurrent hemorrhaging causes disability, persistent pain, and sometimes

death.

In the past three decades, significant advances have occurred in the treatment of hemophilia with the development of plasma-derived and then recombinant clotting factor products. While the development of these new products has proved enormously beneficial, persons with hemophilia continue to face many difficulties that affect their quality of life.

Prior to the development of viral inactivation technologies, many individuals with hemophilia were infected with HIV and hepatitis through their unwitting use of contaminated clotting factor products. The HIV epidemic has cost this community dearly, causing the deaths of over 6,000 hemophilia patients, their spouses, partners, and children.

Today, an additional 2,200 continue to live with HIV and its complications. It is estimated that more than 70 percent of all persons with hemophilia have been exposed to hepatitis C. While the development of recombinant factor significantly improved safety, it is sometimes in shortage and also an extraordinarily expensive medicine, especially for individuals with severe

hemophilia who must treat frequent bleeding episodes or who self-infuse prophylactically as often as three times per week.

Because of the many challenges facing this community and the limitations of current treatment modalities, we look to gene therapy as the most promising approach to cure hemophilia. To this end, NHF has funded numerous gene therapy projects and five scientific workshops on gene therapy, and two-day gene therapy symposium is planned for our annual meeting in October 2002.

We believe that research into a monogenic disorder such as hemophilia may also lead to progress in the treatment of more complex disorders, such as multi-gene inherited disorders, as well as cancer.

In 1996, an NIH report on gene therapy recognized hemophilia as one of the most likely disorders for which gene therapy will succeed. We believe that this statement is as true today as it was six years ago.

In the fall of 2001, vector biodistribution studies from a factor IX deficiency gene therapy trial noted the presence of AAV vector in the semen of a trial participant. That study

also noted that while vector was present in the seminal fluid, there was no evidence of transduction of sperm.

On November 17, 2001, NFH's Medical and Scientific Advisory Council, or MASAC, reviewed this issue and drafted its Recommendation No. 127, which was approved by the NHF Board of Directors the following day, and is attached to this document that you have before you.

The recommendation requests, in reviewing such unexpected findings, that the Recombinant DNA Advisory Committee, or RAC, and the Food and Drug Administration consider the risks to the trial participant and, following appropriate analysis, allow trials to proceed if such risks can be mitigated.

NHF believes that a case-by-case evaluation of unexpected findings will permit improvements in safety and efficacy while enabling continued pursuit of improved treatments for hemophilia.

NHF believes that the AAV factor IX liver-directed gene transfer trial currently being conducted should continue. As Steven Faust, a person with severe hemophilia and co-chairman of

NHF's Advocacy Committee, stated this January before the RAC, we see no inherent risks in these findings that might cause additional risk to the trial subjects.

NHF does support, however, increased patient education and efforts directed at improving the informed consent process, mitigating the risk of potential germline transmission through the use of sperm banking and requiring the use of barrier contraceptive methods, and long-term surveillance of trial subjects via PCR vector dissemination studies.

Indeed, we believe that through this surveillance, we might learn valuable information about the natural history of AAV shedding that could prove useful in future gene therapy trials. NHF's MASAC has laid out detailed guidelines for the conduct of gene therapy trials in its Recommendation No. 120, dated August 16, 2001, a copy of which is also furnished to you.

NHF respectfully suggests that the Advisory Committee consider these guidelines when considering future gene therapy trials.

NHF is heartened by the preliminary results of gene transfer in humans. We are further

encouraged by the prompt review of the RAC and FDA's Biological Response Modifiers Advisory Committee to the vector biodistribution studies, as we believe that this phenomenon may occur in other trials.

We share your commitment to ensuring patient safety, and appreciate your vigilance on behalf of all persons enrolled in gene therapy clinical trials. On behalf of the bleeding disorders community, we urge your continued support for these trials. If enrollment is further delayed, how will we determine if this gene transfer method offers the promise of a cure?

Once again, we thank you for this opportunity to address you on this important matter.

DR. SALOMON: Thank you very much, very nicely articulated.

I think it is always reasonable to point out when you hear something like that, that it is very important for the committee to consider whatever decisions we make affect a group of stakeholders, in this case the hemophilia community, as well as the public, and that is always important to hear that in these sort of

public comments and think about it.

The next speaker is Dr. James Johnson, who is identified as a hemophilia patient. That must have been one of the first two patients to receive the Avigen vector.

Welcome, Dr. Johnson.

DR. JOHNSON: Dr. Salomon, Committee, good afternoon. I am Dr. James Johnson. I am from Edmund, Oklahoma. I appreciate the opportunity to speak to you wearing several hats. I am an emergency physician practicing in Cushing, Oklahoma, a husband and a father. I have lived for 45 years with hemophilia B, factor IX deficiency. In addition, I was blessed to participate in the Phase I safety trial of the gene therapy program at Children's, not this one, but the prior one with the IM injections two years ago.

My participation began in May of 2000. As a side note, I dubbed myself Lad Back No. 6, since I was the sixth person in that study.

As a 45-year-old living with hemophilia and as a participant in a gene therapy study, I want to express my deepest hope that you will allow the current study to continue. This research is of vital importance to the hemophilia community and to

me personally.

When I was born in 1957, it took two years for my parents to receive a diagnosis for the bleeding I had. Because my head was swollen at birth, my parents were told I had hydrocephalus and would be mentally disabled.

Once the diagnosis of Christmas disease was made, my parents were told that I wouldn't live past 20 years of age. I kind of overdid that.

My early treatments consisted of ice packs, splints, slings, and rest. Later, I received infusions of whole blood and fresh-frozen plasma. Finally, when I was 12, I received the first dose of factor concentrate. That was 1970. I promptly came down with hepatitis B, and although I did not know it at the time, hepatitis C.

Through the 1970s, I would go to the doctor and often have to be hospitalized when I needed treatment. Finally, in 1979, I started self-infusion, which is now the standard of care. I was one of the blessed minority that was not infected with HIV, but as I said earlier, I do have hepatitis C.

Also, I have suffered the ravages of arthritic complications of hemophilia as you might

have noticed when I walked up. When we first went to the hemophilia meetings, my wife said, Jim, they all walk just like you do.

As you can imagine, hemophilia has affected every area of my life. Like all hemophiliacs, I have had to deal with educational issues, work and employment issues, and struggles with insurance. From a young age, I knew that I would not be able to hold down jobs that required great physical strength or endurance. Fortunately, I am able to hold down my ER job and even do some extra weekend work.

About nine years ago, I found that I had a hemophilic pseudotumor in my abdomen. This was the result of repeated bleeds in the psoas muscle. There have occasionally been rebleeds which are very painful and often require hospitalization. This happened just this past week and for a while it looked like I wouldn't be able to make it today.

I tell you this not for your sympathy or to act macho, but to let you know that even with today's best treatments, problems still arise.

It has been said of the hemophilia community that we desire to be cured, we don't need to be cured. Everyone is entitled to their own

opinion, but I believe we need a cure. Sure, there are treatments available, but who likes to have to give himself I.V. injections every time he feels pain come on, or injections for days to try to get over a bleed like the one I had this week?

We have treatments for other diseases, but still work very hard for a cure. Does diabetes not need a cure? Does hypertension not need a cure? Does asthma not need a cure?

As for the current gene therapy study, the one I was in, I enrolled about the time one of the other programs lost a patient. Once the program started back up, I, along with my 11-year-old daughter and I, all had the procedure explained to us in great detail. We were told of all the known risks, as well as the theoretical risks that they could imagine.

We were given ample opportunity for questions. In short, I can say from the perspective of both the study participant and as one who has been involved in doing clinical research myself, their informed consent procedure was impeccable.

Every step along the way, I have been informed of any new developments. We are still

given every opportunity to ask questions or check in to see how things are progressing. I have always been and always know I will be able to speak to anyone involved with the program with any concerns or ideas.

I have met with all of the people involved with the program at Chop, from Dr. High, the director, to Dr. Mannow, to Amy Chu, the clinical coordinator, and to even lab personnel. They are committed and responsible people.

I understand that there is concern about the possibility of the AAV vector being present in the semen of some of the participants. This was one of the risks that was reviewed with us before I participated in the earlier trial.

It was always stressed that participation in the study was voluntary, there was obligation to participate or continue even after starting the study. We were informed that I should never expect to father a child after the study because of that risk of AAV infection.

This was fine with us as my wife had already had a tubal. Those that might consider future children were given the opportunity for sperm storage. All of my body fluids were tested

for weeks afterwards. There should be plenty of other subjects like myself who do not plan any further family. They would be able to be in the studies at no risk to anyone else until more is known about germline transmission.

This is an extremely important area of study. Germline transmission is likely to be an equal problem for all gene therapy if it turns out to be a continuing problem here. The hemophilia community, after having gone through hepatitis B, HIV-AIDS, and now hepatitis C, is a very tough, resilient, and responsible community.

We have been on the forefront of the use of barrier contraception to prevent HIV infection, so the idea of contraception and when appropriate, sperm banking, is not foreign to us. Those that do not like that option will not opt for the clinical trials, as did my own brother.

In closing, I want to thank you for the opportunity to speak to you. When I did my senior paper in college, over 20 years ago, I wrote of the potential that gene therapy would one day hold for curing hemophilia. It is here. It is a reality.

As a member of the hemophilia community, I ask you to work with the gene therapy program and

the community to make gene therapy research program safe and successful in the least time possible.

When my daughter asks me about her children and hemophilia, I want to be able to tell her that we have the answer. Please don't make me tell her that we got close, but some minor glitches, whether AAV or AV, stop the program. Therefore, her sons will be at risk for the same difficulties I have gone through.

Thank you.

DR. SALOMON: Thank you very much, Dr. Johnson.

The last speaker in the public comment period is Dr. Kenneth Chahine, Avigen Vice President for Business Development and Intellectual Property.

DR. CHAHINE: Good afternoon. My initial goal was to try to bring a literative perspective to the committee, but after the last few speakers, I think that is not something I am going to try to do.

My goal here today is to first present what we can reasonably glean from the data presented by my colleagues and also to present the assumptions that form the basis of our proposal to

this committee. In our proposal, also, we keep in mind the questions that the FDA posed to the committee.

The second goal is to communicate our proposal and the rationale for that proposal. So, what do we know and what can we reasonably assume?

The first point is that the procedure is well tolerated, as Dr. Kay indicated. There have been no risk to the patient apart from this inadvertent germline transmission risk that we are talking about today.

The second point, which has been clearly talked about amongst the committee members, is the predictive value of the animal models with respect to inadvertent germline transmission. Clearly, some of the animal models don't mimic the human biology, while others may, although even the rabbit dose and clearance times seem to be different from what we are seeing in the first two patients.

The one consistent trend, however, is that in all of the animal models, the vector is either not there or it has cleared over time.

The third point is that the motile sperm fraction may be positive as the dose increase, and will almost certainly take longer to clear, so we

want to make that assumption. I think it is important for the committee to just look at that, and I think our discussion today has brought that out, that the fractionation procedure may not be adequate to address it.

The next point is somewhat of a practical point, is that the current rate and current clinical hold triggers, the Phase I trial is going to take very long to complete, and while in no way does this point alone justify recommendation to continue, it does have practical consequences for Avigen, the scientific community, and the hemophilia population.

The next point addresses one of the questions that the FDA posed to the committee, and that is, should the enrollment be limited to patients or subjects which are unable to reproduce.

Certainly, that will cause delays given the size of the hemophilia population, but will also, in the male population, talking about males that have undergone a vasectomy, limiting enrollment to this patient population will deprive us, the FDA, the scientific and medical community, of the data that we so desperately need to answer the very question we are here today convened to

address.

Finally, higher doses my yield therapeutic levels of factor IX based on the preclinical data. It is important to keep in mind that the primary purpose of this trial is certainly safety, but as Mark Kay pointed out, at the higher doses we do expect, based on the preclinical animal data, to get a dose that is potentially therapeutic.

We hope that this discussion in the future will actually focus more on a risk-benefit as opposed to simply the risk, which is what we are discussing today of inadvertent germline transmission.

So, keeping these points in mind, why is informed consent a reasonable and prudent safeguard against inadvertent germline transmission?

The subjects are already counseled and educated on the potential of not only transient but permanent germline transmission. The patients are already advised to use barrier contraceptives, and as we have discussed here and at the Recombinant Advisory Committee meeting, the risk of inadvertent germline transmission is low.

The subjects are constantly monitored for positive semen and positive motile sperm, and

germline transmission, very importantly, can be completely avoided by banking sperm.

So, in our opinion, when we take the low risk of inadvertent germline transmission, the small number of subjects in the trial, the active use of barrier contraceptives and the sperm banking, these factors together reduce the risk of inadvertent germline transmission to acceptable levels especially when we weigh the risk against the enormously valuable data we will be able to collect, at no risk to the subject, and the potential benefit to the hemophilia community.

So what we propose is the following.

That Avigen should continue its assay development and preclinical studies in various animal models. While there is some question about the validity of some of the animal models that we are studying, I hope it is clear that Avigen is not trying to skirt or avoid this issue, but we are aggressively going after an answer.

The informed consent should be reviewed and updated as needed to reflect the current data, as we have between the first two patients.

The Phase I trial should be allowed to continue regardless of whether the motile sperm

fraction is positive, and that just goes back to the difficulties that we have had in the motile sperm, and once we get to, let's say, the next dose, we are assuming that we are going to actually get some contaminations that are going to give us a positive signal.

The subjects should be monitored until three monthly semen samples are negative. That is currently in the protocol already. We should, even though the value of the fractionation procedure is a question, we want to continue to find out and maybe improve, as has been suggested here today.

We also want to ask a question that is very important and the committee has raised today, which is whether the vector sequences in the semen actually represent biologically active vector. That is just not very clear.

If we can do this and continue the trial, we can use the clinical data to identify predictive inadvertent germline transmission preclinical model, which will help if this trial hopefully goes through later phases, having a predictive model would be very good and clearly for other AAV trials coming in the future.

We want to continue to encourage subjects

to bank sperm prior to the treatment, and the subjects will continue to be informed of their semen results and counseled about whether they should continue to use contraceptives.

The final point is important because it is one of the questions that was raised by the FDA to this committee, which is what happens if you have persistent germline transmission.

We think, and I believe that the committee believes, that the possibility of that is low, we feel like we need to have a contingency, and we believe that if the subjects and the partners are encouraged to undergo counseling by the study physician on a regular basis if the vector is persistent in the motile sperm, that that will mitigate against the risk of transmission.

We selected greater than one year. That was somewhat arbitrary. We are certainly open to discussing with the FDA what would be a reasonable time frame, but that is just the one we selected that we thought was reasonable.

In closing, we are convinced that there is a solution to the time of the completion of this Phase I trial, and are committed to working with the FDA to find and implement such a solution.

Thank you.

DR. SALOMON: Thank you very much.

The schedule shows lunch. I think you may have intuited that I wasn't planning on lunch, and just go into answering the questions. I just wanted to make sure that that was okay with everybody.

Committee Discussion of Questions

We have a series of questions. I am very comfortable that we have set most of the intellectual background here to do this. The first question:

1. If vector sequences are detected in the motile sperm fraction of clinical trial subjects, the current approach of the FDA is to suspend accrual to the study -- in other words, put it on a clinical hold, and that is what happened in this case, just to make sure that everybody is clear about that, these guys can't go forward right now -- regarding the persistence of the vector then becomes the criteria upon which the clinical hold is raised or not raised.

If they are getting out of 14 weeks and they are still positive in their second patient at the lowest dose, that is how they are calculating

that it would take five years to do the study, and it might even be longer.

So I think most of us who have done clinical trials are sympathetic with that being very difficult.

Enrollment has been allowed to proceed when there are data to show that it is negative. In other words, three consecutive samples.

A. Does the committee agree that a clinical hold is warranted when motile sperm tests positive for vector sequence or should enrollment be allowed to continue with appropriate modification made to consent documents?

Discussion?

Let me just point out something here. There is a little bit of a load in here because we are talking about, at least for me, this is motile sperm tests. I am underwhelmed with this motile sperm test thing. In other words, I think it is great science, but I am not sure, and welcome discussion on that point, that you really have to do motile sperm tests here, and I think it would be just easier to do semen.

Go ahead. You wanted to make a qualification?

DR. KAY: [Off mike.]

DR. SALOMON: The point is that you are still on hold. I think that is the main clarification.

Yes, Tom.

DR. MURRAY: First, just to clarify a factual question. The reason you are underwhelmed by the motile sperm test, I am sorry it's unfair to ask Dr. Salomon a question. I begin with a clarification from you.

The reason you are underwhelmed with the motile sperm test, maybe I misunderstand the nature of the test, but if I understood it correctly -- the first thing we are after is if we are concerned about germline transmission, we don't want to see altered genes to a child, and there are a variety of ways to sort of protect against that.

The motile sperm test, as I understood it, was imperfect for a variety of reasons, but most of them had to do with the fact that you might still get AAV DNA even if it wasn't going to be, so it's a sort of both and test.

If it came up negative, would you be pretty reassured that you weren't going to get? Okay. But if it came up positive, you still

weren't sure whether it meant that it was infectious or not. Okay. So, I have got that clear. I wanted to be sure I got that clear.

Let me start then from the back end. If the concern is to prevent the transmission of altered DNA to offspring, there are a number of ways to try to achieve that. One is to test motile sperm. If it comes up negative three times in a row, we feel pretty comfortable that it is not going to happen.

I noticed, by the way, that one of the spokespersons for the hemophilia community actually said about requiring barrier contraception. I am assuming the FDA is not in a position to require it or enforce a requirement for barrier contraception, but I think we need to take that as an expression of the genuine concern on the part of that community.

So, a second order would be to again strongly encourage barrier contraception, provide very clear informed consent. Give the kinds of warnings that Dr. Gordon was, I think, alerting us to, say, look, a way around this is not to do ICSI with your current sperm after intervention, and be very clear about the variety of things that they