DEPARTMENT OF HEALTH AND HUMAN SERVICES

FOOD AND DRUG ADMINISTRATION

CENTER FOR BIOLOGICS EVALUATION AND RESEARCH

BIOLOGICAL RESPONSE MODIFIERS ADVISORY COMMITTEE

OPEN SESSION

Meeting #32

Friday, May 10, 2002

8:10 a.m.

Hilton Hotel
Gaithersburg, Maryland

PARTICIPANTS

Daniel R. Salomon, M.D., Acting Chair
Gail Dapolito, Executive Secretary

MEMBERS

Katherine A. High, M.D.
Richard C. Mulligan, Ph.D.
Mahendra S. Rao, M.D., Ph.D.
Alice J. Wolfson, J.D. (Consumer Representative)

TEMPORARY VOTING MEMBERS

Martin Dym, M.D.
Jon W. Gordon, M.D., Ph.D.
Thomas F. Murray, Ph.D.
Terence Flotte, M.D.
Eric T. Juengst, Ph.D.
R. Jude Samulski, Ph.D.

GUESTS/GUEST SPEAKERS

Valder Arruda, M.D., Ph.D.
Linda Couto, Ph.D.
Mark Kay, M.D.
Stephen M. Rose, Ph.D.

FDA PARTICIPANTS

Jay P. Siegel, M.D.
Philip D. Noguchi, M.D.
Daniel Takefman, Ph.D.
Anne Pilaro, Ph.D.

C O N T E N T S

P R O C E E D I N G S

Opening Remarks

DR. SALOMON: Good morning, everybody. Welcome to day two of the Biological Response Modifiers Advisory Committee Meeting No. 32. I guess we should call it 32B. We have got a title. I have been complaining and I finally got what I wanted a title for these meetings. This one, this is good - Vector Pellucida 2002. Not my title, but, you know, you can't criticize it, I got what I wanted. Thank you.

So, welcome everybody. Today we have changed the scenery around the table quite a bit. So, to get reoriented, I think we should go back around again this time and introduce ourselves, so that both the audience, as well as each other, has a little sense of who we are and what we are doing.

Just if you can introduce yourself, we will just go around the table and give a few sentences on where you are from and what you do, what kind of expertise you bring.

In front of you is a button on the thing. It says speaker. If you push it, it turns red. Talk, and then when you are done, turn it off. Otherwise, there is a funny feedback. So if I am

ever looking at you, gesturing, it means to turn it off. It is one of my big duties.

Introduction of Committee

DR. DYM: Martin Dym, Georgetown University. I worked on the testis and specifically on spermatogonia, which are the male germline stem cells.

DR. FLOTTE: I am Terry Flotte from the University of Florida. We have been working on AAV biology, AAV vectors and AAV gene therapy.

DR. JUENGST: I am Eric Juengst. I am in the Department of Bioethics at Case Western Reserve University and recently rotated off the RAC is where my last connection with these issues.

DR. MURRAY: I am Tom Murray. I am from the Hastings Center, Bioethics, the world's first bioethics research institute, and my work has been in a variety of issues, but quite a lot in genetics, parents, and children.

MS. WOLFSON: I am Alice Wolfson. I am the Consumer Advocate. In this incarnation, I am a policyholder's lawyer representing policyholders against their insurance companies when they don't pay what they are supposed to pay.

In my previous incarnation, however, I am,

and was, a women's health activist and a founder of the National Women's Health Network.

DR. RAO: My name is Mahendra Rao. I am in the Intramural Program at the National Institute on Aging. I am also a member of the BRMAC. I work on stem cells, most parts of the body, I guess.

DR. SALOMON: Jude, we missed you the first time around.

DR. SAMULSKI: I am Jude Samulski from the University of North Carolina, and work in the area of AAV vectors.

DR. SALOMON: I am Dan Salomon. I have the pleasure of chairing the committee today. I am from the Scripps Research Institute in La Jolla, California. I work on cell transplantation, particularly islet cell transplantation and tissue engineering and therapeutic gene delivery.

MS. DAPOLITO: Gail Dapolito, Center for Biologics. I am the Executive Secretary of the committee.

DR. GORDON: Jon Gordon from Mount Sinai School of Medicine. I make a lot of transgenic mouse models of disease and gene therapy for disease. I was on the RAC. I am actually the first person to say the word "transgenic," if that

means anything.

DR. SALOMON: It means a lot.

DR. PILARO: I am Anne Pilaro. I am an expert toxicologist in the Division of Clinical Trials at CBER. I regulate a lot of the gene therapy protocols, in fact, I think I have 167 active right now.

DR. TAKEFMAN: Dan Takefman. I am a gene therapy product reviewer with the Division of Cellular and Gene Therapies, CBER.

DR. NOGUCHI: Phil Noguchi. I am director of the Division of Cell and Gene Therapy at CBER.

DR. SALOMON: Welcome. We will be joined a little bit later by my colleague to the right, Richard Mulligan from Harvard Medical School.

This is interesting for two reasons. One is that this is kind of a revisit to a very important area that the BRMAC dealt with, not the last time, but I guess at least two times ago, where we initially talked about how to address potential regulatory issues specifically with this Avigen trial, and then more generally with how to deal with the potential of infection germline in this case with semen.

We got into the whole discussion about

semen versus infecting the motile sperm and what was the evidence, if any, that you could really infect the germline, the spermatogonia, or infect the sperm themselves, and very much tried to deal with some of the practical issues of what you would demand of any company of a sponsor in doing this kind of research, and to do it in such a way that you wouldn't put an unnecessary hold that could therefore interrupt a very important trial unless there was awfully good evidence.

It is also very interesting in that it is an interesting theme for the two days. In some way I am sorry that some of you weren't here yesterday where there we were really talking about another kind of germline transfer issue, the injection of ooplasm into oocytes for infertile women, but it is an interesting thing now to go on to the idea of potentially doing something like this through therapeutic gene delivery.

We have to read the conflict of interest. Gail.

Conflict of Interest Statement

MS. DAPOLITO: I would just like to read for the public record, the conflict of interest statement for today's meeting.

Pursuant to the authority granted under the Committee charter, the Director of FDA Center for Biologics Evaluation and Research has appointed Drs. Terence Flotte, Jon Gordon, Eric Juengst, Thomas Murray, Daniel Salomon, and Jude Samulski as temporary voting members for the discussions regarding issues related to germline transmission of gene therapy vectors.

Dr. Salomon serves as the Acting Chair for today's session.

To determine if any conflicts of interest existed, the Agency reviewed the submitted agenda and all financial interests reported by the meeting participants. As a result of this review, the following disclosures are being made:

In accordance with 18 U.S.C. 208, Drs. Terence Flotte, Jonathan Gordon, Daniel Salomon, and Jude Samulski were granted waivers permitting them to participate fully in the committee discussions. Dr. Richard Mulligan was granted a limited waiver for this discussion which permits him to participate in the committee discussion without a vote. Dr. Katherine High recused herself from this committee meeting.

In regards to FDA's invited guests, the

Agency has determined that services of these guests are essential. The following interests are being made public to allow meeting participants to objectively evaluate any presentation and/or comments made by the guests related to the discussions of issues of germline transmission of gene therapy vectors.

Dr. Valder Arruda is employed by the University of Pennsylvania. He is involved in the studies of adeno-associated virus vectors. Dr. Stephen Rose is employed by the Office of Biotechnology Activities, NIH.

In the event that the discussions involve other products or firms not already on the agenda, for which FDA's participants have a financial interest, the participants are aware of the need to exclude themselves from such involvement, and their exclusion will be noted for the public record.

With respect to all other meeting participants, we ask in the interest of fairness that you state your name, affiliation, and address any current or previous financial involvement with any firm whose product you wish to comment upon.

Copies of these waivers addressed in this announcement are available by written request under

the Freedom of Information Act.

As a final note, as a courtesy to the committee discussants and your neighbors in the audience, we ask that cell phones and pagers be put in silent mode.

Thanks.

DR. SALOMON: Thank you, Gail.

What we will do here is begin with an FDA introduction from Dan Takefman, will kind of walk us through some of the key issues that the FDA wants to answer. Remember that part of the dynamic here is that we are an FDA Advisory Committee.

There will be times when we all, certainly myself as a scientist, get really interested in some scientific question, but at some point you will have to forgive me if we steer away from that since, if we are not really answering the FDA's question, then, we are not doing what we are supposed to be doing here.

In the meantime, though, obviously, to the extent that any of these scientific issues are relevant to answering the questions, you know, you obviously are here and your expertise is greatly welcomed.

I guess the other thing, as long as I am

giving an introduction on that score, I will just say that we are going to try and come to consensus on some of these questions, but in some instances, there is no consensus, and there is no effort here on my part to force this group into consensus, so well-articulated, minority opinions or even just where we go, I am sorry, but there is no way we can agree on it, that's the kind of information that we need to pin down.

So it is important for us to make sure that we have represented everything as evenly as possible for the community. The last thing I will say to the audience is that I feel you also are participants in this meeting. This is an open public meeting. That mike in the center is open. I welcome all of you, if you have something to say, to come up during the meeting during discussion and make your points, and we will definitely be here to listen to them and try and make sure that we do an adequate discussion of this.

Dan, you are on.

FDA Introduction

Potential for Inadvertent Germline Transmission of Gene Transfer Vectors: FDA Approach for Patient Follow Up

Daniel Takefman, Ph.D.

DR. TAKEFMAN: Thank you. I would like to welcome the committee and speakers, and thank everyone for participating in today's meeting.

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The topic for today is the discussion of potential for inadvertent germline transmission of gene transfer vectors, and as Dan said, this has been a topic of previous discussions and public meetings. Today, we will be discussing the finding of vector sequences in patient semen and to discuss FDA's current approach for patient follow up.

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Concerns regarding inadvertent germline transmission, or IGLT, are twofold. Societal/ethical concerns are based on previous public discussions and publications in which deliberate germline alteration has been deemed unacceptable.

Additionally, there are potential adverse biological effects, such as genetic disorders, birth defects, and lethality to developing fetus, just to list a few which are also of concern.

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What is the likelihood that IGLT would be

deleterious? Well, retroviruses have been used as tools to investigate the role of certain genes which are important in development. I refer to, in this slide, data involving retroviral insertion to the germline of mice and as a specific example, a retrovirus was used to infect a murine blastocyst. In this case, this infection resulted in a mouse strain with a lethal embryonic mutation, which was induced by proviral insertion into the alpha-1 collagen gene. This mutation was recessive, so that the phenotypic effect required homozygosity.

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So data exist suggesting that in the case of retroviruses, deliberate insertion into the germline may be deleterious, but what about data from preclinical animal studies regarding the ability of gene transfer vectors to transmit to the germline?

Well, the FDA does require biodistribution studies with gene transfer vectors in relevant animal models. These biodistribution studies, performed in support of clinical trials, have shown evidence of vector dissemination to gonadal tissue.

However, in most studies, vector sequences have not been detected in semen samples, and the

point I need to make in regards to these preclinical studies is that they are not always predictive of human experience.

A case in point is today's topic in which vector sequences were found in semen from clinical trial subjects, however, initial preclinical studies, such as those done in dogs, demonstrated no detectable vector in semen.

Again, certainly in today's case, animal studies are not always predictive.

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I would like to give an update on the kind of current active gene transfer INDs we currently have in file just to give you an idea of what is being used in the clinic.

You can see here in regards to retroviral vectors, they are predominantly being used in ex vivo types of gene transfer studies, while adenoviral vectors and plasmids are often being used in direct in vivo type of administrations.

You will notice here with AAV vectors, compared to other systems, FDA has seen relatively few gene transfer INDs. Of the few we have, they are primarily in vivo, localized injection type of administrations.

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I would like to go over some of the factors that FDA considers important for assessing risks of inadvertent germline transmission of gene transfer vectors.

Certainly, integration potential of the vectors is important to consider. Of the current vectors being used in the clinic, FDA is considering both retroviral and AAV vectors as vectors with potential to integrate. Certainly with retroviruses, as well as lentiviral vectors, they are known to have efficient abilities to integrate and host genomes.

In terms of AAV vectors, this system is not as clearly worked out as in other systems, such as retroviruses. FDA is currently considering AAV vectors as having a low, but potential to integrate in vivo, and I specifically refer here to a couple of papers from Nakai's lab in which he showed low levels of integration in mouse livers.

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The risk of inadvertent germline transmission is also likely highly dependent upon route of administration. An ex vivo gene transfer would likely represent a minimal risk in terms of

IGLT, while at the other end of the spectrum, a systemic injection would represent a relatively higher risk in terms of transfer to the germline via hematogenous spread.

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As Dr. Salomon mentioned, IGLT has been a topic of discussion, and I would like to go over some of the previous public discussions in order to put today's meeting in a little perspective.

Beginning with the March 1999 RAC meeting, here, there was a focused discussion on preclinical data which demonstrated gonadal distribution. It was the consensus from this meeting that despite this preclinical data, the probability of inadvertent germline transmission occurring during a gene transfer clinical trial was low.

However, further discussion became necessary at the November 2000 BRMAC meeting. At this meeting, we heard data from a trial which involved I.V. administration of a gammaretroviral vector which contained the factor VIII gene for treatment of hemophilia A.

I should point out this was the first trial under IND which involved I.V. administration of a gammaretroviral vector. Data was presented in

which 1 out 12 subjects treated had vector sequences transiently present in semen.

In the one patient, vector sequences were detected at only one time point by DNA-PCR.

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Then, at a recent meeting of the RAC, a trial was presented, which will also be presented today, which involved an AAV vector, which contains the factor IX gene for the treatment of hemophilia B. This is the first trial under IND which involved administration of an AAV vector into the hepatic artery.

Data was presented in which vector sequences were found in semen of the first two patients treated. The first patient had positive PCR signal at multiple time points for up to 10 weeks post administration, and the implication here is that all patients treated in this trial may test positive for vector sequences in semen samples.

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So to summarize some of the consensus from these public discussions, there was a consensus from the RAC meeting on preclinical data that the probability of inadvertent germline transmission is low and that the use of a fertile subject

population was acceptable.

From the BRMAC meeting, the committee agreed with FDA's approach to institute a clinical hold when vector sequences are detected in semen samples from study subjects.

There was a consensus from both the RAC and the BRMAC that there is a need to determine if vector is associated with sperm cells. Using fractionation methods, such as density separation, potential contaminating transduced white blood cells can be removed from sperm cell fractions. You are going to hear more later on from Avigen on their fractionation assays.

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I would like to turn now to FDA's approach for patient follow up, which has been modified in response to these public discussions and from data regarding this current trial.

Prior to initiation of the trial, of course, if during preclinical animal studies, vector is found in gonadal tissue, this finding and the potential for germline alterations should be included in informed consent documents.

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As for FDA's current approach for patient

follow up, if semen from clinical trial subjects tests positive for vector sequences, the clinical trial will be allowed to continue, however, FDA will request timely follow-up testing of fractionated semen. As has been in the case in the past, barrier contraception is requested until three consecutive samples test negative.

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Now, if the motile sperm fraction tests positive for vector sequences, FDA will institute a clinical hold and subject enrollment will be stopped until it is determined that the signal from the motile sperm fraction is transient, and specifically, we are asking for serial fractionated samples to test negative three times over three consecutive monthly intervals.

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I would like to turn now to some of the concerns that FDA has. Specifically, the finding of vector sequences in semen may become more common. Certainly with subject from trials involving systemic or intrahepatic administration of AAV, such as in this trial, every patient treated might have vector sequences found in semen samples.

Additionally, we have new vector classes on the horizon, such as lentiviral vectors, which we know have a high potential to integrate, and there is also new production technologies which allow for higher titer viruses to be produced and new clinical applications of gene delivery systems designed to increase transduction efficiency, all of which may make the detection of vector sequences in subject semen more prevalent in future clinical trials.

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Of particular concern, the fact that patient follow up is difficult with certain populations. Obviously, there are technical limitations in the ability to monitor women and certain men who are unable to repeatedly supply adequate samples. There is technical limitations to monitor these subject populations for evidence of germline alterations.

The specific concern will be re-presented in the form of a question to the committee for discussion in the afternoon session.

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To summarize, FDA's primary concern of inadvertent germline transmission of gene transfer

vectors is with systemic administration of integrating vectors.

A clinical hold is instituted only if vector sequences are detected in motile sperm fractions, and the inability to monitor certain patient populations is a concern and warrants further discussion.

I will end here and just remind everyone that there is a number of background talks and still data on the clinical trial and preclinical studies to be presented, so I would request that we limit the majority of discussion of patient follow up until the afternoon session, but I will be happy to answer a few questions at this time for clarification.

DR. SALOMON: Thank you, Dan.

Are there any questions from the committee to the FDA regarding the overall umbrella charge that we have for today? Okay.

The next are two presentations. It is a pleasure to start with Jude Samulski from the University of North Carolina to talk to us about the biology of AAV vectors.

Guest Presentations

AAV Vector Biology

Jude Samulski, Ph.D.

DR. SAMULSKI: It is a pleasure to be here. I want to thank Daniel for asking me to come up. He requested that I give some type of overview of AAV biology and try to focus a little bit on our understanding of the potential for integration and mechanisms.

I think what I am going to do is offer you an opinion of a consensus of what we think is happening in the field, point you in the direction of probably papers that are relevant, that start to show trends that are happening, but more than likely I am going to end up with the conclusion that Daniel has already described, is that AAV is somewhere on that curve as a vector that can integrate, the efficiency is not well established, but the potential is there.

I will start off by introducing you to the life cycle of this virus. In the laboratory, an AAV particle can have a lytic component or a latent component, so we refer to it as a biphasic life cycle.

It has been established that it is dependent on a helper virus in order to go through a productive lytic cycle, and in this setting, the

virus goes in, reproduces, and progeny comes back out.

What was established in the laboratory in the early seventies was that if you took AAV particles and put them in cells in the absence of the helper, you could see this persistence, what was referred to as "latency," and in this setting, it was determined that the virus was establishing an integration event in the chromosome, and in this integration event, it appeared to be targeting, meaning it was going to a specific locus in the human genome.

This was all done in vitro and tissue culture cells, and to complete the biological life cycle, if you take these cells and now superinfect them with adenovirus, AAV has the ability to come back out of the chromosome and reenter its lytic component.

So in the laboratory, it was established the mechanism in which we could argue how AAV, which was found in nature in clinical isolates of adenovirus, how these two would co-persist, but we could also explain a question of what is the consequences of AAV infecting the cell in the absence of its helper. Is that genetic suicide?

That answer was no, the virus has a mechanism of persistence.

I should argue that there is absolutely zero data of AAV integration in humans. This is all established in vitro, and it is inferred that this mechanism can take place.

I should also mention that the early studies of AAV showing up in clinical isolates, it has only been isolated in adenovirus, although herpes can supply the same helper function. There has never been a clinical isolate of herpes that has had a contamination of AAV.

So what you should be asking yourself is that we can mimic a paradigm in tissue culture and substitute other viruses, but what appears to be out there in nature is this co-relationship. This was established in vitro, and it is presumed that this can also happen in vivo.

The genome is fairly simple. It is about 5,000 base pairs, and what is of importance today is paying a little bit of attention to what is referred to as the Rep genes and the inverted terminal repeats of the virus, which are the origins of replication, the packaging signal, and what appear to be the break points that join

recombination events with the chromosome.

Of the Rep genes that are made, it has been shown that it is the large Rep proteins, Rep 78 and 68, that appear to be responsible for the integration events. I just want to point out that in AAV, these are identical proteins. They only differ by a splice variate, and in the absence of adenovirus, this is the dominant protein that you see in the presence of adenovirus. This comes on first and then it switches over to Rep 68.

They all have enzymatically identical activities. They bind to the AAV terminal repeat and what is called a Rep binding element. They have a site-specific, strand-specific endonuclease activity where they can nick this molecule, and they have helicase activity which allows it to unravel to DNA.

So we see a relationship with the Rep proteins were the key element on the virus, which is the origin of replication, showing that it has a binding site, a nicking site, and enzymatic activities to allow this virus to replicate.

So the first evidence of AAV integrating site specifically was generated in Ken Burns' lab in 1996, and in this study, what they did was

pulled out some junctions, sequenced the junctions, and went back and used those sequences as probes.

This is just a representative example from our lab that shows that if you look at your chromosome 19 locus in a control cell, it is about a 2.6 kilobase fragment, but after you integrate and establish independent clones, you can find variance that show evidence that the chromosome sequence now has a rearrangement suggestive of an insertion, and some of these are multiple fragments showing that there is amplification and rearrangement.

If you take a blot like this and strip off the chromosome 19 probe and then come back with the viral probe, you can see there is co-segregation of these viral sequences with these chromosome 19 rearranged, so this was the data that said there was a preferred site of integration, a rearrangement of chromosome 19 and a co-localization of these sequences with chromosome 19 sequences.

Ken Burns and others looked in detail to bring to try to understand why was this virus going to this specific locus, and from that study came the following information.

There is an identical Rep binding site and a nicking site located on human chromosome 19, so what we had was a mechanism that is virtually of viral origin sitting on chromosome 19, that gave a putative reason for why this site is preferred as an integration locus over any other sequence in the human genome.

What I should point out is that further studies have shown that not only is the Rep binding required, the spacing between this binding site to the nicking site and the nicking site itself, so if you take these sequences and count them up, there are over 15 base pairs.

It is argued that a sequence over 15 nucleotides is only represented one time in the human genome. This is probably why this virus is only targeting this locus. This element is present in about 200,000 copies in the human genome, which would argue that the Rep protein is sitting on lots of spots on the human chromosome, but it is only when it is this context that it can initiate the event to promote the integration step.

So we have a model and a mechanism that is being supported both in vitro and in vivo.

A group in Italy went on to show that the

site has an open chromatin confirmation and that it is not a closed site, so it is not a site that is unaccessible. All of these things are beginning to support the type of DNA structure that AAV needs to see in order to go into the chromosome.

A number of labs, including our own, have gone after looking at these integration events, and most of you are pretty well aware, that if you look at retroviral integration event, it is a fair precise cut and paste mechanism in which it cuts the chromosome, integrates its genome, and there is like a 3 to 5 nucleotide duplication on either side.

When you looked at these AAV proviral structures, what we saw was there were a lot of tandem repeats, amplification events, and all of these things were supporting a type of integration that was completely different than the well-characterized retrovirus integration.

This has been consistent both in cell lines, as well as episomal integration events, as well as in vitro systems, so there is a mechanism for integration that is not consistent with a cut and paste. It is referred to as a non-homologous amplification mechanism.

Our lab and others went on to look at the break points between the viral terminal repeat, which I showed you has this origin activity, and this hairpin structure, and the junctions between that and chromosome 19.

What you can see was there was very little fidelity and conserving the integrity of the terminal repeat. You would get break points that were scattered throughout these hairpins, and these are just positioned here on the sequence to give you an impression that there is no fixed break point between the viral sequence and the chromosome 19. They cluster around this hairpin element, but other than that, you can virtually find break points throughout these sequences.

If you look at that from a biological point of view, it again suggests that AAV may have a problem in retaining its integrity as a virus if it's indiscriminately breaking these hairpins and going into the chromosome, but this virus has a phenomenal ability of carrying out a step code gene correction.

There is technically two copies of every sequence in the hairpin, and since there is two hairpins, there is the total of four copies on the

virus, so between all of these copies, the virus will gene convert back and forth and regenerate these sequences with fair efficiency, so you always get a wild-type virus coming back out even though what is integrated in the chromosome may be somewhat fragmented.

Because the virus also integrates in what appears to be head-to-tail concatemers, it is preserving the integrity of these hairpins internally, and again allowing it to use it as a template to amplify and come back out of the chromosome.

So to get to the mechanism, Matt Weitzman in Roland Owens' lab did an experiment in the early nineties that said that they could show that the Rep protein of AAV could form a complex between the terminal repeat of the virus and this pre-integration site.

Again, this made logical sense because there was the same Rep binding element on both of these sequences. This is just an illustration from Sam Young's data showing the Rep protein bound to the terminal repeats of an AAV vector. It has an extremely high affinity for the sequence and a Rep complex binding to the same element on chromosome

19. It was data like this and other that began to propose a model that the virus express its Rep protein, it binds to this element on chromosome 19.

In vitro, Rob Cotton showed that this is sufficient to start a synchronized single-stranded DNA replication. So now you have this region of chromosome 19 serving as an origin. Since the Rep protein is terminally attached to this chromosomal sequence, and you can reinitiate, we feel that there is a number of initiation events that are taking place on this region of chromosome 19.

It should be understood that there is an enzyme called Fen-1 which is a host enzyme, that actually repairs this type of repeated initiation event, however, if you have a hairpin or a protein attached to this, it doesn't have the ability to correct these sequences.

So what happens is you see recombination events taking place to resolve these molecules. It has been suggested that the AAV genome, which has Rep, allows for Rep-Rep tethering mechanism, as Weitzman showed, and at this point it is all host enzymes that are involved in inserting this sequence into the host genome, and this type of tandem repeat, head-to-tail type of format.

This is data that was provided to me by Regina Hildabraun. It is not published. It is coming out in a journal Virology. She has developed a real-time PCR assay to look at the efficiency of AAV viruses to go to chromosome 19. It is a PCR assay that look at the terminal repeat and a locus on chromosome 19.

What I think is important to see here is that she can score integration events taking place over the first 72 hours or so, but the most important thing is that the wild-type virus, which she is seeing an integration event for about 1,000 particles, so it is suggest about 0.1 percent of all the AAV virus is capable of carrying out integration.

This is completely different than like the retroviruses where it is 100 percent integration.

As Daniel said, there is a propensity for the virus to integrate. The efficiency is what needs to be look at in this setting.

This is a paper that was published by Ernst Winocour. I think this is of importance because what I am going to suggest to you is this is another parvovirus called minute virus in mice. It's an autonomous parvovirus. Nowhere is its life

cycle does it establish latency. It has no mechanism. There has never been any data supporting it.

But what Ernst was able to do was show that these viruses also have terminal repeats, they also have Rep-like proteins, and that he could take an episome substrate and show that this virus could also integrate into a target sequence if the Rep protein on this minute virus was present and if the subsequent sequences were available.

So what I think this is suggesting is that the parvoviruses have proteins that are involved in replication that are able to carry out nicking and helicase activity on substrates. In the case of minute virus of mice, there is no target in the genome.

In the case of AAV, there is an origin identical to AAV sitting on chromosome 19. So the question may be, does AAV really set up a latency or is this an interaction between Rep proteins and target sequences, and 1 percent begins to suggest that it is not a very efficient mechanism.

I am going to shift gears and now talk to you about vectors because I think this is where most of the interest is. In the laboratory, a

number of people generate vectors by different procedures.

In our lab, we use plasmids to start to make the vector, so now we only retain the terminal repeats. The gene of interest is in the middle. You have a helper plasmid carrying the Rep and capture genes, and another plasmid carrying the essential sequences from adenovirus to activate all of these steps.

What happens when all of these are in the cell, you produce a single virus particle, which is an AAV particle carrying the foreign gene of interest. If you take these viruses and put them in tissue culture cells, and put them under selection, what you see is if you go to the chromosome 19 region and look at individual clones that had the vector integrated in the human genome, you don't see a significant rearrangement under chromosome 19 sequence.

So unlike wild type where it appeared that 70 to 90 percent of the integrations were targeting this locus, the vectors have lost this ability to go to chromosome 19. It has been shown by a number of labs that if you add Rep back to this reaction, these vectors will go to chromosome 19 and

integrate.

So it is fairly well established now that AAV vectors have no targeting capacity and that what they do have is the capacity to integrate into the chromosome under these selected conditions.

This is an approach that Charley Yang took in the lab about seven years ago, in which he made AAV vectors that were carrying a plasmid origin and ampicillin sequence, as well as a selectable mechanism to look at selection in eukaryotic cells.

He made this into a virus, allowed it to integrate into the chromosome, and he used enzymes that were cut outside of the viral DNA, closed this up into a circle, and pulled out these so-called cellular junctions, and when he characterized these, he came up with the following results.

The break points of the terminal repeat and the chromosome were almost identical to what we saw with wild type. They clustered around the hairpin structure, but there was no defined break point in any of these vectors.

When we looked at the location that they were going into, they appeared to be random on chromosome 17, 7, 1. We had two examples of it integrating on chromosome 2. But what we were

seeing was that all of the characteristics of integration were identical to wild type. It is just that their targeting ability was lost. Instead of going to 19, it was random.

If you look at the vectors, they were again consistent with this head-to-tail mechanism and amplification event or rearrangement event. I should mention that David Russell has just published a little paper in Nature Medicine that has shown another clustering of these things pulled out of HeLa cells, and we have generated the exact same information. There is breakage and duplication and some type of random repeats that are being generated.

So I want to point out because I think we get misled a lot when we think about AAV's integration and that it is something special. This ability to form concatemers is something that was documented a number of years ago by Schimke's lab. In fact, if you look at any transgenic animal that has ever been generated, it is always generated in a head-to-tail concatemer formation.

If you look at virtually any cell line that is established by plasmids to give stability, it is typically a head-to-tail concatemer, that is

going into the chromosome. So what we see is that AAV is probably using host enzymes to generate these concatemers that eventually go into the chromosome.

As I mentioned to you, without the Rep protein, there is no targeting capability. This integration appears to be random. The insertion that takes place at the integration site is not a cut and paste mechanism, it's a deletion, amplification, rearrangement, illegitimate type of recombination.

This is just our data showing all of the break points that we have generated both with vectors with wild type AAV as far as the junctions that are generated between the terminal repeats and the chromosome, and you can see that again there are preferred clustering sites, but there is no distinct break point that takes place between AAV molecule and the chromosomal DNA sequence.

We concluded from this study that when AAV vectors go into cells, it is cellular recombination pathways that are responsible for the integration of that, and that there is no viral participation in this enzymatic step, it is all carried by cellular recombination.

If you look at the data that has been generated, it falls under the category of an illegitimate, non-homologous recombination. This would be true if you put in plasmid DNA, oligonucleotides, any piece of DNA that ends up going into the chromosome. It is following a pathway that supported cellular enzymes carrying out the integration step.

I want to just summarize this and then I am going to switch to the last third of the talk, which is going to just talk about information generated with vectors in animals.

Right now, AAV vectors do not target chromosome 19. They are identical to wild type with respect to the terminal repeat break points. They are essentially identical at this level. The head-to-tail orientation of vector proviruses, you can find tail-to-tail and head-to-head, but this is pretty much the dominant species you will see.

They rearrange to chromosome integration site. There is not a cut and paste mechanism. There is always some type of deletion, amplification, and rearrangement that takes place at the integration locus.

So by all these criteria, AAV fits the

conditions of an insertional mutagen. It has the ability to go into the chromosome, and the critical question is at what frequency does it carry out this insertion event.

This is where I think we began to accumulate data in the field that drifted us away from all that information that was derived in vitro, and you should understand that the data was derived in vitro was under selected conditions with a gene, such as G418 or neomycin, so that you are only looking at the integration events.

In vivo, the first data that began to suggest that this may not be consistent with what was happening in vitro was actually carried out in Terry Flotte's lab where they were looking at adeno-associated viruses in monkeys after administration for airway gene delivery.

When they characterized this, they saw that the virus was persisting for a period of time and the virus could be rescued completing all of those steps that we talked about in the life cycle, but it was showing up as an episome. There was very little data suggesting that this type of persistence was taking place as an integration event.

This is a paper that I would like to direct people to, because I think buried in this paper is some really important information. This was a study carried out in Jim Wilson's lab where what he virtually did was an in vivo selection like what we do with in vitro selection with G418, in an animal model that had a disease for the liver, so the AAV vector was transducing a gene and to deliver, that he could put a selective pressure on.

This selective pressure meant that if this liver was to survive, the virus had to integrate. After it integrated, you could see nodules begin to grow of liver cells. He characterized those nodules. He showed they had integration events in them. They were similar to what I have just described for in vitro.

They were tandem repeats, rearrangements, and an illegitimate recombination mechanism, but if you go into the paper and dig at the multiplicity of virus that he was putting into the liver, 1012 particles per liver, he was only getting about 0.1 percent of the liver cells showing an integration event.

So I think what Daniel was referring to is where does AAV fit on this curve of an obligated

integration event versus the potential to integrate, and this study, under selective pressure, there was a frequency that was derived, which I think may be telling to the type of numbers that may happen in the absence of selection.

I point to these last two papers only because it has been characterized in extensive detail in muscle, and I bring up Phil Johnson's study because he now has an abstract that is going to be presented as ASGT, where he is showing that a majority of what I think he calls 98.5 percent of all the vectors that are in skeletal muscle are persisting in episomal form.

He does a real-time PCR assay. I am not going to try to describe his data, it is written in an abstract form, but I think it is something that the field in general will want to look at and see if this will be something that can be used for other target tissues.

But it is consistent with the theme. What I did not talk about here today was any of the data that Mark and Kathy have generated, because I know they are going to speak later and they can tell you specifically what has been derived in their hands, but I think the theme is we see what these vectors,

they have the propensity to set up a persistence, the data that has been generated in liver, muscle, lung, and brain is that episomal forms that are predominantly seen, but there is always the potential and evidence for integration.

This is the last paper that I am going to point you to, and I am going to just mention this because I think this is going to give us a starting place to begin to understand AAV integration in whole animal.

Terry Flotte and his lab have generated some data showing that the DNA-dependent protein kinase, the gene that has mutated in SCID mice, seems to have an impact on the molecular phase of AAV genomes.

Again, I am going to paraphrase what Terry's data says, and he can speak to it in more detail because he has got new data that is a little bit more extensive. It appears that if you knock out this protein kinase, which is involved in immunoglobulin rearrangement as one example of its role in the human cell, the virus appears to integrate more efficiently into the chromosome.

This is an enzyme that plays a role in end-to-end joining, and it seems that if you lose

the ability of these host enzymes to form the so-called concatemer structure that we all characterize, you can see an increase in integration event takes place.

So it appears that if you are defective in one pathway, AAV will just follow another host mechanism for persistence, which is an integration mechanism.

Again, if there are any specific questions, I will ask you to direct them to Terry where he can give you the details of what is going on, but what this data tells me is that we probably we will be able to identify these so-called cellular recombination pathways that are influencing AAV vectors when they go into so-called non-dividing tissue.

I am going to conclude by trying to reemphasize the following points. Wild type and AAV vector integration is not very efficient, and this fairly well documented in vitro. It is something that seems to be a theme that is recurring in vivo.

If you look at the ability of the virus to target chromosome 19, it is absolutely dependent on a viral protein called Rep. The mechanism is now

well understood because they are identical binding sites to facilitate this targeting.

AAV vectors, which do not have Rep protein, do not have the ability to go to chromosome 19 into the site-specific manner. If you look at the proviral structure of wild type AAV and vector DNA, they are essentially identical at all levels.

The break points and the terminal repeats, the amplification, the concatemerization, and the rearrangement under chromosome sequence is identical whether it's on chromosome 19 or randomly inserted throughout the genome.

Finally, with the limited number of studies that are being done, it appears that in non-dividing cells in vivo, the AAV vectors exist predominantly in an episomal form, and again, I will conclude.

Daniel basically summarized the AAV field by saying it has the propensity to integrate into the chromosome, where it fits on that rheostat as being very efficient or not efficient, I think it is going to be dependent on more studies in vivo in which we can continue to accumulate data.

But as of today, what we keep seeing is

some propensity for this episomal form, but the risk is still there, and I will stop there and take questions.

DR. SALOMON: Thank you very much. Very interesting.

Q&A

I have a couple of questions that kind of occurred to me in the setting of thinking about this thing riskwise. You have been very straight about it. What is interesting is a lot of times when it is introduced for the first time, people talk about OAB, it's a parvovirus, it has been in humans for a really long time, and it has been extremely safe in the sense that it is not associated with any known disease entity, and the implication is many times that therefore, AAV gene therapy as a vector is going to be similarly safe.

However, I think what you very clearly point out in all the molecular biology that has been done with the vector is that an AAV vector really isn't anything like a wild-type AAV in the sense that now what you have got mainly is episomes, it is not integrating in chromosome 19, so there is a lot of assurance that one might take from the first part of the data that it is probably

not reasonable to carry forward into thinking about AAV vectors.

DR. SAMULSKI: Right. I will give opinions on both sides. I think if you look at the biology of the virus, it falls in the biological features, so that we don't see significant immune response generated from AAV infections. You don't see that with wild type.

You don't see the virus taking over the host cell as a lytic virus does, so there is consistency in that aspect of saying AAV is more like its features of being non-pathogenic, but I think you only need to hear what Phil and them mentioned at the RAC probably every time AAV is discussed, you know, this is not normal. You are putting in 1012 viruses into a focal injection, hundreds of particles, lots of genomes. This is something that doesn't happen in nature, and so it shouldn't be considered as the viral life cycle, because in that setting, we can't reproduce the viral life cycle. We are not getting a systemic infection that is disseminating and maybe setting up latency.

We are inducing an artificial way of getting persistence. So I think you are right on

the money there. I think what will go back and forth between these systems is how much does the vector mimic wild type. As far as integration they are identical, it is just one is on 19, the other one is random.

So there is some ability to go back and forth as to what is happening.

DR. SALOMON: So the second question I had was I don't know a lot about chromosome 19, so I apologize for what I am certain are stupid questions to the geneticists here, but is it clever that the virus chose this area in chromosome 19, is that a safe area to integrate in that?

I guess the follow-up question here would be maybe one thing to think about, has anyone thought about it, is if you add the Rep gene back and let it integrate into a place that we know is safe instead of having all this episomal DNA that we have no idea what it is doing.

DR. SAMULSKI: Your question is something that you would discuss at a cocktail hour, why does AAV go to 19. We could say mechanistically, there is a viral origin sitting on 19. Did the virus pick it up from 19 and retrofit it into its life cycle or is that a remnant, some integration event

that took place who knows when.

It is only conserved in monkeys and humans, so it is a sequence that is not found, so there may be some selective pressure for why that took place. Is it a safe site? In tissue culture, we are in HeLa cells, there are 19 chromosomes, 3 copies in 19, we can get latency all the time. In vivo, there hasn't been the kind of studies you would want to see, and if AAV integrates in 19, is that going to be an adverse event.

I would argue 19 in liver cells may not be essential, but 19 in another tissue like neuronal cells may be essential, but to get back to your question, which I think is more directed to what is on that locus, there is no gene located at that region.

Michael Linden has argued that there is a transcript that can go through this region that is related to a muscle transcript, but from our and other studies, there has never been an integration event that has disrupted that gene or the potential for the gene, but again, there are all tissue culture cells, so I think it is an interesting biology.

When we first saw this, what is clustered

on chromosome 19 were a lot of genes we would have liked to have seen it go into, the receptor for polio virus, a receptor for a lot of other viruses, and we thought, oh, maybe, AAV will integrate, give the host cell a mechanism of protection from another infections agent, and there would be a reason for why it targets, but this locus is not by those type of genes, although it would have been a nice story. So it is an unknown.

DR. SALOMON: I had one last question, and that is when it integrates and then almost sort of kind of does its version of concatemerization in that area -- that is not quite exactly what happens, but -- what does it do to the promotor regions in the ITR, is the payload gene still promoted, or does it destroy the promoter region, so you basically have dead genes there?

DR. SAMULSKI: AAV is not like the retrovirusus where it has a promoter, a strong promoter in the LTR. It has promoter-like activity, but all the cassettes have the promoter built in between the terminal repeats, and so the gene remains intact, the break points seem to be in this buffering area in the terminal repeats.

So, again, all of these things are skewed.

They are put under selection so you insert the genes that go in intact, and they rescue them out. We can only see the products that E. coli will tolerate, so you have to realize that head-to-head and tail-to-tail formations are not very stable in E. coli, so we are getting a biased opinion every time we pull these out.

The PCR reaction is extremely biased because that is Mother Nature's best primer, it's an 80 percent GC hairpin structure. If you try to prime through that region, you will generate deletions, so we even think a lot of our data showing break points is an artifact of pulling out junctions.

The only data that begins to support that if you have a real controlled Rep expression, you don't see as much amplification rearrangement. The group in Italy put the Rep gene on the regulatable promoter, and they actually dosed in the amount of Rep, and what they was the integrations were more well behaved.

So I would say that we have not been able to mimic what probably the virus does very well, but we can score all the downstream events. It goes in a chromosome, it looks like this, and so

forth.

So I would be hesitant about taking my opinion about this field and turning it into this is the fact of all it all happened.

For the vectors where there is no Rep, and you do see the integration, it is cellular mechanisms that are putting it into the chromosome.

DR. SALOMON: Dr. Rao and then Dr. Mulligan.

DR. RAO: Is there any evidence of mobilization of the integrated thing, wild-type infection?

DR. SAMULSKI: That is a good point. There is the risk of mobilization if you get an added infection and a wild-type AAV infection, so you need a two-hit kinetics to move the vector out of the chromosome.

In the laboratory, if you do those experiments, wild-type dominates the product that comes out, because there are more elements that ensure packaging, and they are not in the vectors, but you do mobilize it if you get a two-hit kinetic.

DR. RAO: Is there a rough percentage on that? I know wild-type predominates, but --

DR. SAMULSKI: Wild-type plate 90-something percent of all the virus that comes out, and if you cycle it, it is the only virus that you see. The vector doesn't compete very well in that setting, but the risk is there, in an in vivo setting.

DR. MULLIGAN: In the in vivo case, the integration question is complicated by all the free copies, and I think it is important that people that are not experts here get a sense of if you had very efficient integration in the sense that you had one copy for large number of cells, but then you had hundreds of unintegrated copies, that would confuse your interpretation, so can you characterize for people how you get at the issue, that is, if you just look at the sum of unintegrated copies, and that is a large number, and then the sum of integrated copies, and that is a small number, then, one conclusion is that you have mainly unintegrated gene transfer, but in principle, on a cell-by-cell basis, you could have very efficient integration, while on top of it you could have a large amount of unintegrated copies.

Now, in vitro, I know that is not the case because you can actually directly assess that, but

how have the various tests actually ruled out that that is not the case?

DR. SAMULSKI: I think that is a good and hard question. I think Mark has generated data that begins to look at that where he has put virus in hepatocytes, and he will probably discuss this, and then did a partial hepatectomy to let the liver cells grow, and tried to score how many of those regenerated liver cells still carry a copy suggesting that that fraction had integration, and the ones that lost it were primarily episomal.

I will let him describe that, but I don't think there is any good way to assess that question.

DR. MULLIGAN: I would think that now that there is these, in human cells, outlaw PCR approaches, the question is can you actually directly calculate the total absolute number of integrations independent of how much total DNA is there?

DR. SAMULSKI: I don't know how I would do that. I think this is what Phil Johnson is doing in his abstract. He is looking at ALU real-time PCR going across genomes and stuff like that.

DR. MULLIGAN. Has anyone looked, like

Ernest Whittaker, like his system if you have an adeno-infection or HIV infection, and you all of a sudden do an AAV infection, is the propensity for integration of AAV into, say, HIV, a higher integration because it's unintegrated initially than it would be to go in the chromosome?

DR. SAMULSKI: I think that is another good question, that is, if you are in a cell that has substrates, what is the fate of AAV to those substrates, will it go into them, or a more preferred event. I don't think anyone has an answer to that, but it's a good question. It is something that has got to begin to be looked at.

I think I would like to just emphasize that AAV in the early days was put in the bone marrow stem cells with a lot of efficiency, and then it was shown that as you tried to amplify these cells, they weren't very good and I think it was speaking directly to the fact that it wasn't integrating and therefore, you could transduce them and get positive cells, but once they are asked to divide, you lost that.

So I think why AAV has been such a niche virus for the so-called non-dividing cells is because is can set up this persistence. I think

the integration frequency is probably going to be determined by do non-dividing cells carry out illegitimate recombination, at what rate compared to a dividing cell. That is going to be an important number that is going to influence the outcome in these type of studies.

DR. GORDON: I have a couple of very quick questions that are just simple factual answers.

Where in the life cycle of AAV does the uncoating of the genome take place? That is one. The second question is you said that when you add Rep back to the vectors, then, you get chromosome 19 integration again. How is it added back, as a gene or as a protein?

DR. SAMULSKI: The answer to the first question is the parvovirus are argued to go into the nucleus and uncoat to release their DNA into the nucleus. There is probably a capsic component still associated with the virus that is sitting on those terminal repeats that either prevents it from, you know, being naked DNA, but at the same time may recruit other factors to the origin.

As far as the second question that you had -- I forgot it already --

DR. GORDON: Adding Rep back.

DR. SAMULSKI: That's my senior moment there.

Rep protein has been added both as plasmids, as physical protein injectate, and as inducible protein in the cell line, and all of those will take vectors and allow it to go to chromosome 19.

The last thing I will mention is that both the Italian group and our lab have generated a mouse that carries the chromosome 19 locus, and in our case, it is sitting on the X chromosome. When we put wild-type virus into that, it goes to that chromosome 19 locus even though it's on the X chromosome, again suggesting it's the cis elements that are driving where it goes, and not that it happened to be on 19 in humans, and stuff like that.

DR. DYM: I think you alluded to my question, but i am going to ask it anyways. Can you clarify or comment on the ability of the AAV to get into dividing cells versus non-dividing cells, and, of course, in the testis, the spermatogonia are very actively dividing, the sperm are not.

DR. SAMULSKI: I think there is no difference between AAV going into dividing or

non-dividing cells. If the receptor is present, it will bind, and then I think the mechanism for internalization is clathrin-coated pits, endosome release, and traffic.

If you can carry out those steps, it is indistinguishable whether it's a dividing cell or non-dividing cell. In the very early days, it was suggested that AAV preferred dividing cells, but that was in vitro looking at selection and therefore you were biasing the system.

I think once people went in vivo, they realized that all of that was probably misleading a little bit.

DR. MULLIGAN: You didn't mention about other AAV serotypes, so in principle, the efficiency of the intervention would depend upon just the virus titer.

Do you have any sense that AAV-1, for instance, which in muscle is much, much more efficient, would potentially be better at infecting germ cells?

DR. SAMULSKI: I think Richard's point is a really interesting one because we and others have seen that the other serotypes have better propisms, are more efficient. The question is what are their

integration mechanisms.

The only one that we have data on is Type 4. Type 4, which is camana monkeys, will target monkey cells and integrate, will target human cells and integrate in the chromosome 19, so the wild-type virus will capitulate exactly what the human virus is.

The other four, 1, 3, and 5, it is unknown, but they are so homologous, about 80 to 90 percent homologous, they all bind to the terminal repeats, they all can package each other's DNA. Chances are they will do the same type of integration.

There are differences in these terminal repeats if you look at them. Type 5 is different than Type 2, and if that is a substrate, that may be more prone for recombination enzymes, you may see an integration frequency that is different.

DR. MULLIGAN: I just meant the capsid, looking at risk for germline infection, if it happens just proportionately, it much better infects that cell and even though integration is very efficient, then you get more efficiency.

DR. SAMULSKI: I misunderstood. I think if the virus has a more efficient tropism in those

kind of cells, chances are the integration frequency is going to be higher. That is kind of a given.

DR. SALOMON: Sort of a follow-up question here is -- and you may have answered this, and I apologize if you did -- if you have a cell that is actively dividing or is activated, let's say, so it has a lot of open chromatin structures, it is more likely to integrate in that setting than in, let's say, a stable cell that is not activated?

Obviously, where I am going is in, you know, if you had an injury or inflammation, or something, are those areas in which the rules might be different?

DR. SAMULSKI: Sure. I think that is exactly what the data are supporting. This virus looks for open chromatin contacts. Events that were scored appeared to be in genes, promoter regions in the gene. I think they are all because of the same reason, these were open chromatin. If it's condensed chromatin, there is probably no mechanism, because again it's a cellular event and it is going to be acting on cellular regions of the DNA, better accessible.

DR. SALOMON: That was great. Thank you.

DR. SAMULSKI: Thank you.

DR. SALOMON: Very useful.

The second presentation is on germline transmission by gene transfer vectors and some thoughts on assessing the risk from John Gordon, Mount Sinai School of Medicine.

Germline Transmission by Gene Transfer Vectors

Assessing the Risk

Jon Gordon, M.D., Ph.D.

DR. GORDON: I was asked to talk a little bit about not necessarily what we are doing to address this problem in my own lab, but just to talk about what I think are the points of susceptibility for germline integration of vectors into various gametogenic cells and to review the literature on it, so that is what I will do.

I am not an embryologist by profession, and I don't wear the lot on spermatogenesis either, but we have a spermatogonium expert in the audience in case I make a mistake, so that will be good.

The ontogeny of gametes in relation to their susceptibility to gene insertion. Primordial germ cells are the cells that ultimately arise to both eggs and sperm, and these arise in the yolk sac or the epiblast in the mouse at about three

weeks' gestation in the human.

There aren't a very great number of those. They then migrate by ameboid movement through the dorsal mesentery to the genital ridge. During this migration process, they also multiply. These cells are quite easily identified because they stain very strongly for alkaline phosphatase.

They arrive to the genital ridges that may be the end of five weeks' gestation in the human. During this period, the cells are unprotected, that is, they are not within the capsule of a gonad, and they are mitotically active, allowing infection by agents that require mitotic activity. We will return to this point of what agents may require it.

Fetal gene therapy must take this risk into account, and the RAC had a sort of mock fetal gene therapy protocol presented one time, and this issue has to be raised.

Now, female gametes, which are of a little bit less interest today, but they are important, of course, they become oogonia, and they divide by mitosis until about 5 months or a little longer to generate several million oogonial cells. At this point, many begin to die, while others become primary oocytes.

Primary oocytes enter meiosis, a complete crossing over, and then they stop. The chromatids remain associated, but crossing over is completely. Then, they are surrounded by follicle cells in what are called primordial follicles.

Once they are in the primordial follicle, they become relatively inaccessible because you have to get through the layer of follicle cells, which is a single cell layer basically at this point, in order to reach the egg, which is sitting at the end of crossing over in the so-called dicteate [ph] stage.

They sit in this stage until the follicle begins to develop towards ovulation, and there is some hypothesis that this long term association of the chromatids has something to do with chromosome nondisjunction in older eggs.

Now, at puberty, the follicle develops in response to FSH from the pituitary. Numerous follicle cells surrounding the oocyte are within the follicle wall, and they begin to produce glycoprotein "egg shell," the zona pellucida.

So, as the egg is developing, then, the number of follicle cells that sit between the egg and the outside world increase, the wall of the

follicle becomes a consolidated structure, and the zona pellucida is laid down. This is a glycoprotein human egg shell, mammalian egg shell, very hard to penetrate.

As the follicle matures, meiosis resumes, and one resumes, and as the first polar body is released, the chromosomes then move to a metaphase of the second meiotic division, and that is how they are found after ovulation.

To enter the egg, genes must past through the follicle wall, they have to get through or between the follicle cells around the egg, and then they have to get through the zona.

We would regard the egg as a non-meiotic cell at this point.

At ovulation, the egg is in metaphase II and is surrounded by the zona and the granulosa cell layer. Some of the cells are ovulated with the egg.

Although immunoglobulin molecules will pass through the zona, there is no evidence that naked DNA or viruses will do so. There have been experiments at least with retroviruses that have no viruses that I am aware of where very high amounts have been put onto zona intact eggs, and then lacZ

staining look for later in cleavage, for example, without seeing anything.

After fertilization, MII is completed with release of the second polar body formation and formation of the female pronucleus.

Now, micromanipulation to assist reproduction can assist genetic material in by passing the zona. I just would like to make the point here of two contrasting papers in the literature, one by an Italian group in I believe now the late eighties, in which they asserted that if you performed in vitro fertilization with plasmid DNA sitting in the medium, about 30 percent of the mice born were positive for transgene sequences.

The plasmid they happened to use in this case was a commercially available SV40-based vector and to prove that they had integration in these mice, they cloned the material back out of the mouse genome and sequenced the vector material that was in the mouse genome.

The published sequences contain nothing junctional, they were all internal sequences to a commercially published sequence. They also did a so-called MBO1/DPN1 digest to show that the

material was in mammalian cells and was therefore digestible with I believe it's MBO1, if I don't them in backwards order, and the only problem with this southern blot showing disappearance of this band was that the southern blot did not include the molecular weight size that the band was originally in.

It stopped before you could get that high up on the gel, which wasn't very high, I might add, about 4.3 kb.

So, needless to say, there were a few weaknesses in this publication. Nonetheless, it made the cover of Cell and was accompanied by a very exuberant editorial saying that this had something to do with evolution, plasmids jumping into gametes out there in the ocean where fish have ex vivo fertilization, for example, and multiple labs tried to repeat this work and 2,300 mice were produced in a number of labs, we tried it too, could not reproduce this work even using the identical reagents, and no one makes transgenic mice this way even though it is a heck of a lot easier than microinjection.

However, if you do another experiment, and that is, mix plasmid DNA with sperm, as was done

before but now inject the sperm into the egg, so now you are bypassing the zona with a microneedle, and the sperm and DNA around it go into the egg, a significant percentage of the mice are transgenic, and that is a reproducible result.

So, in humans, if we think about micromanipulation, and this is something I have been asserting in an editorial that I have in press, we have to think about the fact that the environment had better be clean, because we can get DNA in by that method.

My opinion of what occurs here is that the pronucleus forms quickly after the sperm is injected, DNA gets entrapped into it, and it is pretty much the same as microinjecting DNA into a pronucleus.

Now, another interesting point is there is there papers indicating that retroviruses and lentiviruses will infect MII oocytes, which are not meiotic reactive, but which do not have a nuclear membrane. The chromosomes are sitting at a metaphase of the second meiotic division to produce transgenic cattle, monkeys, and mice.

I think these papers are very interesting, but there is one slight problem with the assertion

that it is the non-meiotic MII oocyte that is the target, and that is, of course, that if you soak MII oocytes in the vector, and then fertilize them, there are still going to be vector around after fertilization, and it is not really possible to completely clean them and then fertilize them to show that you had no vector around at fertilization, so it is possible in my view that fertilization occurred and then these vectors went in.

But, nonetheless, you can get MII oocytes transduced with retroviruses and in mice, now lentiviruses from David Baltimore's lab, and again this raises an issue in clinical in vitro fertilization where the zona is opened not infrequently, either for injecting sperm, for biopsying embryos, and so on.

Now, male gametes. Now, in the male, the primordial germ cell step is the same. They get to the genital ridges as before, but them they become dormant where they are contained within sex cords. They sex cords are like the future seminiferous tubules of the testis, they remain this way.

The sex cords have a membranous barrier between them and the outside world, but this is

much less protected structure than it becomes after puberty. The cells are mitotically inactive and relatively unprotected.

At puberty, these PGC's become spermatogonia and begin dividing. Type A spermatogonia are renewable stem cells that produce more Type A spermatogonia, but they can also produce Type B spermatogonia, and those are committed to meiosis.

It has been shown, mainly by Ralph Brimster's lab, that spermatogonia can be transduced with retroviruses and lentiviruses, I believe are correct now. This is one in vitro and it is not clear how efficiently one could accomplish this in an intact testis with intact spermatogenesis. Perhaps our colleague in the audience, an expert on spermatogonia, can speak to that, but it clearly is biologically possible to transduce them even though it is not very easy.

Generally, they are put back into a testis that doesn't have its own spermatogenesis, so that you can sort of have a natural selection for those cells exposed to the vectors in the outside world, and you can get transgenic mice that way.

Now, when meiosis beings and the

spermatogonia are formed also, the testis becomes organized the seminiferous tubules. Pre-meiotic cells are at the tubule periphery where agents can get to them, but they will have to get through the tubule wall, but theoretically, they could be reached from a hematogenous spread to the seminiferous tubule.

However, Sertoli cells, situated within the seminiferous tubules, form tight junctions that sequester meiotic cells behind what is called the "blood testis barrier," so actually not a barrier between the blood and meiotic cells, it is between the Sertoli calls and the meiotic cells.

Sperm move toward the lumen of the tubule as they complete meiosis and morphological transformation. Now, this barrier is needed, of course, because it doesn't occur because these meiosis-specific proteins don't appear until after puberty, and therefore they are potential immunogens, so this has to be a immunologically privileged site, and that is the rationale for having the blood testis barrier.

Meiotic cells are difficult to access except retrograde through sex ducts. You can inject vectors into the epididymis, for example,

and find them in the testis. So someone is undergoing, for example, prostate gene therapy, it is not at all impossible that one could get vectors moving retrograde back up and thereby get to the cells that are behind the blood testis barrier.

Male gametes. Now, sperm maturation or spermiogenesis, is characterized by a loss of most cytoplasm, replacement of the histones by much tighter binding protamines, and near complete cessation of gene expression. I say "near" because there are a few post-meiotically expressed genes.

Again, what you have to realize is that the idea of sexual reproduction is to give all gametes an equal chance of getting to the egg, and if you have postmeiotic gene expression could have allelic variance which would give sperm an advantage theoretically, and so the organism does everything possible to prevent that.

As meiosis begins, actually, once Type B spermatogonia become committed, these cytoplasmic bridges remain between the cells. These are very large and they allow even mRNA size molecules to pass from one cell to another, so allelic variations between spermatogenic cells, those differences are minimized in terms of their

potential impact on spermatogenesis, and then late in spermiogenesis, there are a few genes active, but mainly there are the chromatin is very tightly condensed and very difficult to access.

I should point out parenthetically there that there have been papers from Anderson's lab way back when, showing that retroviruses like open chromatin in preference -- or DNA hypersensitive chromatin -- in preference to highly condensed chromatin.

The nucleus then becomes surrounded by what I would call the giant lysosome, the acrosome, contains lytic enzymes for presumably digesting your way through the zona in fertilization, and it is difficult to access DNA in the sperm head.

Now, again, I would say that there are some papers saying that this has been done successfully. There is a paper from France saying that pig sperm can be transduced with adenovirus. This paper found lacZ expression in cleaving embryos after exposing sperm to adenovirus, and then found piglets that had mRNA-derived by RT-PCR that had mRNA derived from adenovirus in multiple tissues of these piglets.

Now, I would just analyze this paper a

little bit for your benefit, if I might. The lacZ vector used in that paper was a vector that was received from another laboratory and which had a nuclear localization signal. So the lacZ should have been in the nucleus of these embryo cells, and indeed, when we have used such things on embryos, we see the nucleus stain.

However, the pig embryo is loaded with lipids, and they are basically black. You can't see the nucleus in a pig embryo, and if you want to inject a pronucleus in a pig to make transgenic pigs, you have to centrifuge the embryo to get the lipid out of the way, so you can even see the structures.

So, in the photograph showing lacZ staining of these embryos, there were black embryos that were exposed to the vector, and there were slightly less black embryos that were not exposed to the vector, and the nucleus was not visible in either case.

The staining for lacZ was done for 15 days in this experiment, and I would assert to you from my own work with lacZ staining that you could stain your teeth if you did it for 15 days.

The staining was on the zona. There is no

reason why there should be staining on the zona, but we have used lacZ staining on embryos with adenovectors on zona-free embryos just exposing the embryo, we never seen staining, not on zona-free, but, for example, injecting it under the zona, we never see zona staining.

These people found RT-PCR-positive tissues in all three germ layers of the piglets born, that is, ectoderm, mesoderm, and endodermal derivatives. Now, this vector was replication-defective. The only possible way to be in all three germ layers is if it integrated and got replicated.

However, their southern blots were negative. To me, that is a very incongruous result, so I don't believe the result, let me just give you my own opinion there.

We tried this in mice and could not repeat it, at least in mice. However, I think this paper and the other paper with the sperm-mediated plasmid transfer speaks to one of the sort of difficult problems for the FDA, I believe. These are published data and it is very difficult to say, oh, well, that's great, but it is not a good paper, so we will just ignore it. It is very difficult to ignore it when people say they are doing these

kinds of things successfully, then, one has to step in and address it.

Male gametes continued. Now, the mature sperm on route to release can be exposed to vectors via fluid from the seminal vesicle, prostate, and in the urethra, a small amount of urine, as well, although maybe you are uncomfortable to see or hear that, it's true.

Virus found in the ejaculate could be from any of these four sources or from the sperm themselves if somehow it got there, and I should say that one could imagine all also that the cells that line the sex ducts could be received vector from the bloodstream and then pass it on theoretically to sperm although I think that is very unlikely.

As vectors diversify, though, we can't completely rule that out. Reports of successful transduction of mature sperm are difficult to repeat, and I have already discussed that.

Male gametes continued. When sperm bind to the zona, they undergo the acrosome reaction. The acrosome reaction is fusion of the outer acrosome membrane. You remember the acrosome is the giant lysosome. The best way to think of this,

as I have told my family, it seems to work on them, if a fist put in a pillow, a soft pillow, and that put into a garbage bag.

Now, the soft pillow is the acrosome, and the fist is the nucleus, so the nuclear membrane is coming in contact with the inner acrosomal membrane. Then, you have the feathers, which is the acrosomal contents, then, the outer acrosomal membrane, the other side of the pillow, and then that is right underneath the plasma membrane, the plastic bag.

Well, if you slash open the plastic bag and the outer side of the pillow, and sew those seams together, you will release all the feathers to the outside. The acrosome reaction occurs, and the bottom line of that is a lot of the sperm plasma membrane is lost.

So even passive association of genetic material with the membrane, a lot of it can be lost. However, often the entire sperm is incorporated into the egg and the plasma membrane and components associated with the tail may still be there, so it is possible to passively get it in, I think.

Now, shortly after fertilization, sperm

head decondenses to form the male pronucleus. DNA replication begins. Genetic material that enters the egg with sperm, as I pointed out, from these microinjection of sperm experiments, you can have a relatively highly frequent integration.

Now, the early embryo, I wanted to mention it because of my allusions to IVF, the early embryo cleaves within the protective zone until implantation, when hatching occurs. Now, hatching and implementation virtually occur concomitantly under normal circumstances, so the embryo is difficult to access even though it has to get out of the zona.

However, micromanipulation can open the zona and expose the embryo to gene transfer agents for more extended periods. Take, for example, the many thousands of IVF cycles that go on every year where the zona is open to theoretically assist hatching. In my opinion, assisted hatching is of debatable effectiveness, but there have been some papers that embryos from older women implant more frequently if you open the zona, and what happens there is you may open the zone at the four-cell stage, put it in the uterus and it sits there until the blastocyst stage and then implants, and so now

you have the naked cells of the zona opened embryo sitting there where agents that may be in there from the woman being infected with something, from the lab technician who had gene therapy, from whatever source, have a much greater time period in which they could get to the embryo.

The embryo is quite easily transduced by a variety of agents, retroviruses being the first one done by Yenish in the early seventies, recombinant retroviruses in the mid-eighties, controversy whether adenoviruses integrate. Our own lab did one where we did early embryos with adenovirus, and what we found was adenovirus was very toxic, so if you put enough in to be sure of getting transduction, the embryos were all killed. If you put in so little that none of the embryos were killed, you had no transduction, but if you have sort of an intermediate level, then, very rarely you can see PCR-positive tail biopsies in offspring that is clearly a mosaic integration.

So it is possible to infect embryos, and as IVF becomes more and more interested in zona opening, let me give you another example, pre-implantation genetic diagnosis. You may have heard the speech of Frances Collins at the ASGT

meeting in California where he went on about pre-implantation genetic diagnosis and result of finding out things from the genome project, for example.

Well, pre-implantation genetic diagnosis requires first injection of the sperm because if you do regular IVF, there is hundreds of sperm that are still around and many bound to the zona. When you then biopsy the embryo for PCR, if one of those other sperm gets into your PCR reaction, you are looking for one molecule here, that is, or two molecules, to genotype the embryo, an extraneous sperm is unacceptable, so you have to do ICSI, that is, intra-cytoplasmic sperm injection.

Well, that opens the zona, and as I pointed out before, it is very easy to make transgenic mice if you do ICSI with DNA in the medium.

Then, you go back later and open the zona again, but this time a much bigger hole, so that you can take a cell off to do genetic diagnosis, and so I think from the point of view of germline transmission, it is much more risky thing to do than just tell the women to get pregnant. She will have a 75 percent chance then of having a baby that

hasn't have genetic disease in the case of recessive genetic disease. She has a 100 percent change of getting pregnant, of course, while in pre-implantation genetic diagnosis, her chances are only 20 percent. It is going to cost her nothing to get pregnant, while in pre-implantation genetic diagnosis, it costs about $15,000 to get pregnant. Then, she has no risk of all these other things, which, of course, in pre-implantation genetic diagnosis, she has.

I might also add that she has to be superovulated for pre-implantation genetic diagnosis. There have been deaths from hyperstimulation syndrome. There have been problems with surgical retrieval of oocytes. I was a little angry with Frances for always saying that instead of saying how about just doing prenatal diagnosis and doing an abortion in the quarter of cases where it is necessary.

I just thought I would give you a few pictures here. There is spermatogenesis in a normal testis. Actually, it is a seminiferous tubule that we injected with adenovirus vector, and the periphery of the less mature sperm cells. As you see, you move towards the periphery, the sperm

heads become condensed and you can see tails, and so on.

Then, they are released into the lumen of the tubule and then may go out. I said there is minimal cytoplasm on sperm, but a normal variant in sperm is a so-called cytoplasmic droplet, which kind of like hangs behind the mid-piece of the sperm, so there can be a significant amount of cytoplasm in ejaculated sperm.

Here is a developing egg. I was pointing out to you the barriers of penetration of this structure for its virovector. Here is the DA nucleus. You can't see the incipient zona pellucida, but there is a very white band around as it is beginning to form, many follicle cells around, and then the follicle wall. So it is difficult to get there.

This is some experiments we did when injecting adenovirus vector into the ovary at unbelievable concentrations against any for lacZ. You can see that this vector didn't want to get into the follicle. The eggs didn't make it through frozen section, so we have done immunohistochemistry to show that the follicle is not penetrated.

Here is injection directly into the seminiferous tubule. My contention is that we should do provocative experiments that tell us whether or not it is biologically possible to transduce these cells, because in the future, gene therapy will be promulgated, vectors will diversify, their tropisms will change, their structures will change, the methods of administrations will change, and the number of people treated will grow, so we need to know can these things actually get in, not we need to design experiments not to show ourselves as they probably won't happen. We need to do experiments to tell us whether or not it can happen, so that we can write the proper consent forms.

When we do adenovirus vectors into seminiferous tubules directly in a procedure we call seminiferous tubule cannulation, we see a lot of staining for lacZ, this is immunohistochemical, in the periphery, and it looks as if Sertoli cells are the transduced cells.

This is a Sertoli cell. It is sort of anchored to the periphery of the tubule and extends its way in. The Sertoli cell surrounds the spermatogenic cell and sort of helps it complete

spermatogenesis, and, by the way, also concentrates androgens to very high levels in this region of the testis.

We are doing this test to ask ourselves can we transduce these intermediate cells that are behind the blood testis area by injecting vector directly into an intact seminiferous tubule. We believe that this suggests no, but we think we need to go to nucleic acid hybridization to really know because especially like for AAV, which has a delayed expression, we need to know where the genetic material actually is.

This is just a view of the acrosome reaction. This is the acrosome. With those enzymes for getting through the zona pellucida, the main one is a proteolytic enzyme acrosome, and I hate to say this, but there is a paper from Japan where acrosome was knocked out and the mice were completely fertile. It has never been repeated, but everybody believes it. That is rather a shock, I must say.

You can see how much of the plasmid memory can be lost in the acrosome reaction.

That is the summary them of where gametogenesis is more or less susceptible to being

genetically transduced.

DR. SALOMON: Thank you very much, Jon. That was excellent.

Q&A

It is interesting that yesterday, we were talking about a procedure that came very close to what you just described, so what they are doing it taking infertile oocytes from the presumed patient or from the infertile mother, and taking normal donor oocytes and injecting the sperm -- it's ICSI -- but also ooplasm from the normal oocyte donor.

One of the issues that we discussed in detail was the potential of chromosomal DNA fragments being injected with the ICSI, and you have now given additional evidence. We were concerned of recombination potential, the gene delivery.

DR. GORDON: Well, let me just say that I wrote an editorial to Fertility and Sterility, which is in press, but I haven't received galleys yet, and therefore, there is some concerns about it being released to the committee and then, of course, to the public yet.

But I list all these procedures of micromanipulation and their potential risks for

inadvertent germline Transmission. I makes some suggestions about what might be done to sort of do quality control in IVF labs. That would at least address this issue proactively.

I mean should we multiplex PCR media in which we do micromanipulation just to make sure there is not DNA in there, or should we discuss whether or not practitioners of this forms of IVF, we should at least know that they haven't had 1015 retroviruses put into them the day before for gene therapy for something, which could happen down the road.

I think we should at least begin to study this because there are tens of thousands of cycles done.

Now, in terms of the papers of ooplasm transfer, I have a written editorial published, in which I say that the use of germline gene manipulation -- unfortunately, these people did this mitochondrial DNA analysis on newborns who had received ooplasmic transfer, and the found the DNA of the donor cytoplasm in the newborn's bloodstream -- they called this the first germline gene transfer.

Well, of course, these new mitochondrial

DNAs were not transmitted through the germline yet, so it was a little bit of a loose use of the term, and remember that if it is mitochondria, you can always get rid of it is you just allow the person to be a male who has received all of that, because sperm mitochondria are not transmitted to the next generation.

There was a very interesting paper where sperm mitochondria were injected into an egg and destroyed and then liver mitochondria were injected and weren't destroyed, so it seems like the egg knows how to find sperm mitochondria, distinguish them from others and destroy them.

So that type of gene transfer if not germline in my opinion, and although these people wanted notoriety for using that phrase, I am not sure they got the one they were looking for, but in any case, that is very easy to thwart. All you have to do is make sure that it's only male reproduction after that.

DR. SALOMON: This is very interesting but we are going to have to stop, because that, we discussed yesterday. Too bad you weren't here.

I have one quick question and then we will start from the panel. In terms of interpreting

experiments where you say we looked at gene transfer with adenoviral vectors, they were all adeno that you showed us this time, no AAV, right?

It got into the Sertoli cells, for example, it didn't get into the spermatogonia, and from what I looked at, those were spermatogonia, not the more mature spermatids, right, because you were showing right at the edge there --

DR. GORDON: Some maturing, yes, it looked like there might have been spermatogonia. That slide does not rule out. That slide shows that we can certainly get a ton of vector there, which I believe is important. I think provocative tests need to be done, not bloodstream injections where we will never find the cells that got exposed.

DR. SALOMON: The specific question I had is at some point, you point out very well that the DNA in the developing sperm condenses and transcription diminishes dramatically to almost stopping, and I certainly have no expertise in exactly when in the cycle that is happening, but it would seem to me that particularly, experiments done with mature sperm in which you tried to do something that required transcription as the measure of whether you got gene delivered would be

a failure because there is no transcription going on, so even if you got gene in, to just take sperm, incubate it with AAV vector or adenovector or any vector, and then show this is not lacZ positive wouldn't mean anything.

Did I miss something along the line?

DR. GORDON: Well, I am not so sure how much transcription is needed to get that to occur. I mean you are more a vectorologist than myself, but it would seem to me that if you get a vector into the head of the sperm, that the sperm could then fertilize the egg, and then it would decondense into a pronucleus and development would begin, and any vectors that were in there could then act as if they had just infected a dividing cell line.

So, if you could get the sperm to carry it in, you wouldn't have to transduce the sperm, integrate it into the sperm head, but you could certainly get viruses into the embryo by that method theoretically.

DR. SALOMON: Right. So if you want to test it, you would have to test it several steps down the line, that you have delivered whatever you carried in, got transcription again, make the

beta-galactoside gene, then, you do the colored substrate. I am just trying to understand. From what you are saying, if you took just mature sperm and incubated them with a vector, and that might even occur in the -- there is probably a lot of transcription going on in the spermatogonia, though, right?

DR. GORDON: Yes.

DR. SALOMON: That must be a metabolically active cell.

DR. GORDON: Yes.

DR. SALOMON: So this would probably not be a criticism of studies done on the first things you showed.

DR. GORDON: Well, here is what I did. I exposed sperm to adenovirus vectors, made sure that they got exposed to is, 10, 100 virions per cell, and then I did in vitro fertilization with those same sperm.

Then, the embryos that those sperm conceived were evaluated for expression. The other thing we did was we allowed fetuses to be produced or newborns and we evaluated them by PCR.

Now, my opinion is there were a lot of experiments that preceded those in which animals

were injected in their brain with adenovirus and then bred. Well, you know, there is 300 million sperm in a mouse ejaculate, and you are looking at 10 of them when you look at 10 pups. So that is statistically not satisfying.

But if you have an in vitro system where every cell is exposed and then you have a way of assessing whether it got in, I think that you are doing much more to really answer the question.

DR. FLOTTE: I had sort of a natural history question. I was wondering if you had any thoughts about human endogenous retrovirus sequences in our genome and what is the most likely access that those originally had to the human germ line.

Then, a follow-up question, do you think there is any significance to the fact that we don't find human endogenous AAV sequences in the genome?

DR. GORDON: The first question. Well, there is a tiny little sort of moment of accessibility I think at hatching of the embryo in vivo. The embryo has to hatch out and then implant, and it is naked. That could be a point where a person who had a lot of viremia or a lot of virus in interstitial uterine fluid that you could

get one in.

I must say that in mice, retrovirus-like sequences are also found endogenously in the genome. That, to me, would be a logical place to think of it occurring. It is very hard to imagine it occurring. You could also think of a viremic male having it get into a spermatogonia.

I mean now that it has been shown that you can get it into spermatogonia, at least in vitro, it might be much less probable in vitro, but if you have 30 million centuries to work on it, you know, you may see it. So this is exactly the point, of course, about provocative testing, too.

So that is my view. Now, what is the significance of not finding a virus, I mean I really can't say anything about that. It could be a combination of factors - I haven't looked enough, the virus has too low an integration frequency, there is not a biological setting in which there is good access of a virus at a susceptible point, you know, ontogeny, such as uterine fluid at a time of implantation.

So it would only be speculation on my part, I don't know.

DR. SALOMON: Dr. Dym and then Dr. Rao.

DR. DYM: I had a couple of questions, but first I will thank you also for a lucid presentation. I will just comment briefly that there are a number of people who are using in vivo approaches, as I think you know, to get viruses into the spermatogonia through the seminiferous tubular lumens. Brimster is one and there was a paper by Blanchard & Vokalhyde in Biology of Reproduction in 1997.

Again, they showed that it only went into the Sertoli cells, but Brimster and a number of others, actually, five or six labs, in monkeys and in rodents and in cattle, are using this seminiferous tubule injection or ret-A testis injection. It is in vivo, but it is not practical. I mean you can't put it in that way normally.

But this leads me to my second question having to do with barriers. You mentioned barriers. I do believe there are barriers from your work and from other people's work, and that is why probably virus in a muscle or systemic virus may not get into the spermatogonia, but this is in normal animals or maybe in normal people, but the barriers actually break down when there is a diseased person or a diseased animal.

I am just wondering if you know anything about that and if, when the barriers break down. Actually, another thought came to mind. For example, in AIDS patients, the barriers are broken down and the virus, which is circulating in the blood, let's say, from a man who has gotten infected via needle, the virus is in the blood, and then eventually it breaks down and gets into the closed lumen or semen compartments, whether it is testis or epididymis, but it does get across the barrier, so viruses do get across in diseased conditions.

Some of these patients you are talking about might have a breakdown of the barrier.

DR. GORDON: I am glad you actually mentioned that because I think it is worth some comment. First of all, I think viruses might be able to break the barrier and then go through. I mean viruses can hurt cells, and if you flood cells with them, you might get a weakening of a barrier by the very action of the virus.

Then, there are disease states. Disease states are exposed internal portion of the seminiferous tubules to the outside, I think intuitively are not likely to be so flagrant as to

raise the risk significantly just because I think that would have a big impact on spermatogenesis, too, but I did want to say that there are ways -- well, the FDA speaker was point out that localized injection is less risky than perhaps systemic injection, but I think one exception should be taken to that, and that is injections into things like the prostate, which by no means is an inactive area of research, so I do agree that while these barriers exist, one cannot predict from that intuition that in all of the settings of gene therapy, where a vector's ability to cross barriers may vary, or a vector's ability to violate the barrier and get in on their own may vary, where disease states may vary.

So biologically, these barriers exist, but I think it is quite true that you can by no means be guaranteed that they are going to protect you completely, and provocative testing is needed.

DR. RAO: You give a very nice summary, at least for me, in terms of understanding that there is great protection of the male and female gametes.

So, let's say you do, in fact, a patient with adeno-associated virus at some titer, 1011, and now see adeno-associated virus in ejaculate.

What would you speculate as which cell was infected and does it have to actually be an integration event that you are seeing this one year later?

DR. GORDON: No, I don't think it has to be an integration. A year later is really a long time. But weeks later, as what happened in this case that probably prompted this discussion, could be in anything, could be seen in the fluid component, could be in other cells, there is always a few white cells perhaps, could be in the debris that would slough off from endothelium, not at all necessarily in sperm, and even if it came out with sperm, that doesn't mean it is in them. It could be just on them, and washing them could take care of it, or IVF could take care of it.

I think it is reasonable if a sperm fraction in infractionated semen is positive to step back and say, well, now, a red flag has been risen. If you find it in whole semen it really could be from any variety of sources.

DR. DYM: Just one more comment maybe in relation to what you said. You know, those of us who work in the testis, and there are many of us working on spermatogonia who are actually trying to infect and transduce the spermatogonia and the germ

cells, we never think of doing it in the sperm, we always think of doing it in the spermatogonia as the only permanent way.

I think that maybe addresses some point that you made. That would be permanent, you know, generation after generation after generation. It's an eternal cell, it's an immortal cell, the spermatogonia. The sperm dies.

DR. RAO: The reason I asked the question was one needs to evaluate, when you are looking at any kind of risk, as to where the virus particle is present, and that is an important thing that we need to clarify if you are going to say that you detected in the sperm or in the ejaculate where is it really going to be present.

From what we heard, it is unlikely to be present in the sperm per se, at least in the sperm DNA, and given what we have heard about integration events, maybe it is unlikely to be present in the spermatogonia, but we need to know it. It is best to ask the expert directly.

DR. GORDON: Well, I just would say that if you found it in semen a year later, I would be a little more worried that it got into is spermatogonium because, as he said, that is an

immortal cell. Spermatogenesis proceeds in waves, and if you get it into any cell that is not the Type A spermatogonium, you may have its appearance, but then it will disappear.

That is why people are trying to do spermatogonia, but I must add that there are a number of papers in the literature, none of which I believe, but there is man of them saying that you can get DNA into mature sperm by a variety of methods - opening the epididymis and giving it an electrical shock with your biorad electroparator, people will say that works. I mean you should see those data, they are so pathetic, but nonetheless, they are published, so what can you say, the data are published.

DR. SALOMON: I would like to call this session to the break. We will see everybody back in 10 minutes.

[Recess.]

DR. SALOMON: We will go ahead and get started.

This portion of the session, we are going to have a series of presentations from Avigen and then from the University of Pennsylvania.

The next two speakers will provide us some

specific information on the AAV vector from Avigen.

The first speaker is Mark Kay. Welcome.

A Phase I Trial of AAV-Mediated Liver-Directed Gene Therapy for Hemophilia B

Mark Kay, M.D., Ph.D.

DR. KAY: Thank you.

What I would like to do is summarize our Phase I trial of AAV-mediated liver-directed gene therapy for hemophilia B, which is a collaborative effort between many investigators at Stanford, the Children's Hospital, Philadelphia, and Avigen.

[Slide.

Today's focus are issues pertaining to the inadvertent germline transmission of AAV vector and what I would like to do is summarize data related the clinical trial to date.

[Slide.

There has been some discussion about integration of AAV in the liver, and although Jude suggested that I was going to show data about integration, I actually have those slides, but not in this particular talk, so let me just summarize where things are and give some explanation.

We know that, in general, if you inject reasonable high doses of AAV into mice that you can

get something in the neighborhood of 50 percent of hepatocytes that are stably modified with AAV. In some situations, it might be slightly higher or lower.

Now, it turns out that if you give these regular doses of AAV into mice, the vector genomes actually get into almost 100 percent of the hepatocyte nuclei, but over time, most of those single stranded genomes are lost and here is only a small proportion of cells that remain with stably transduced vector genomes

Now, the proportion of integrated genomes is actually small. Generally, it is actually less than 5 percent. I think the definitive evidence that AAV integrated in liver was a study done in collaboration with Linda Couto and Hikiyuki [ph] Nikai, where they actually were able to clone out integration junctions, so basically within the vector, they put bacterial origins of replication and then were able to take genomic DNA, put them back in the bacteria, and clone out the covalent linkage of the vector where it integrated into the genome.

Now, this was a very useful technology, but it does not quantify how much integration

actually occurred. So we have recently published on studies where we have injected AAV into animals and we wait for a period of time until there is stable transduction, and then what we actually do is a hepatectomy.

Now liver cells will equally regenerate, such that each cell divides once or twice, and as a result, DNA genomes that are not associated with centromeres or telimeres are lost, and we have positive and negative controls for this, and what we find is that in most situations, the amount of integrated genomes, of the stable genomes is very small, it is usually less than 5 or 10 percent of the double-stranded vector DNA.

Now, gene expression from the integrated forms, which again is small, and the episomal forms, parallels the proportion of vector DNA in each state, so if you do a partial hepatectomy and you look at the amount of vector genomes before and after, you get around 90 to 95 percent reduction both in gene expression and in number of genomes, again indicating that most of the expression comes from the episomal forms.

There is no detectable increase in the proportion of integrated genomes over time, and