# FOURTH NATIONAL GENE TRANSFER SAFETY SYMPOSIUM: SAFETY CONSIDERATIONS IN THE USE OF AAV VECTORS IN GENE TRANSFER CLINICAL TRIALS

March 7, 2001

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# I N D E X

I N D E X (Continued)

# P R O C E E D I N G S

## WELCOME AND INTRODUCTION

DR. PATTERSON: I think we will get started right now.

(Slide.)

Welcome to the Fourth National Gene Transfer Safety Symposium. As I think all of you are aware, our topic for today is "Safety Considerations in the Use of Adeno-Associated Virus or AAV Vectors in Clinical Gene Transfer Research.

I would like to just take a few minutes out of what is a fairly packed schedule today to review with the context, rationale and agenda, as well as the goals of today's symposium.

(Slide.)

This is the fourth in a series of symposia. They are part of the overall DHHS initiative to ensure the protection of research participants. This effort was launched in March of last year and these symposia are intended to provide public forums for expert review of emerging scientific, medical and ethical issues in gene transfer research.

The symposium, however, apart from these over arching and lofty goals, have some very specific and practical goals. First, they are intended to enhance our understanding of both the safety and toxicity of this research. They are intended to help us identify critical gaps in what we think is our current knowledge of the field. They are intended to maximize the safety of people who volunteer to be research participants in these studies and they are intended to enhance the informed consent processes for these individuals.

Lastly, we hope that they optimize the development as well as the day-to-day conduct of these clinical trials.

(Slide.)

A good question for today, though, is why a safety symposium on AAV vectors and why now? Well, one impetus for today's symposium is what we see as an increasing interest in the use of AAV vectors. I will touch on each of these things briefly and they will be gone into in much greater depth by several of the speakers today. But, as many of you are aware, AAV vectors offer a number of useful features for gene transfer and recent improvements in production methods have only heightened interest in their use in a variety of clinical applications.

The second impetus for today's meeting, however, is the emergence of some recent concerns about possibly some tumorigenic potential in a particular animal model of a certain disease. And we will hear more about that later today.

(Slide.)

In terms of the useful features of AAV as a gene transfer vector, it is derived from what is considered to be a nonpathogenic virus that is designed not to express viral proteins and, therefore, is thought to be unlikely to induce a host immune response.

The vector is capable of effectively transducing a wide range of target cells, including nondividing target cells making it a useful vector in clinical applications that involve cells in a resting state.

And, finally, the vector can under certain circumstances achieve long-term transgene expression.

(Slide.)

This is an electron micrograph showing AAV particles along with a much larger adenovirus particle. As many of you are aware, some of the older methods for production required coinfection with adenovirus in order to yield an AAV prep and, therefore, required elimination of purification of the contaminating adeno prior to final vector lot preparation.

(Slide.)

More recent methods, however, involve cotransfection of a plasmid bearing the necessary Ad helper genes and thereby eliminating the need for Ad infections and the subsequent difficulties of eliminating contaminating Ad from the final vector prep.

(Slide.)

But despite these useful features and recent improvements in production methodology, adenoviral -- AAV trials still account for only about two percent of all gene transfer studies registered with the NIH.

(Slide.)

This small percentage notwithstanding, there has been a recent, albeit modest, increase over the past couple of years. The first AAV trial was submitted for RAC review in 1994 and over the past two years we have seen, well, what is really a doubling every year, although the numbers admittedly are still quite low but there is a rising interest.

(Slide.)

There has been a total of ten trials submitted for RAC review and they are graphed out here according to clinical indication. You can see that the majority of them or half of them, five of them are devoted to the study of cystic fibrosis, two for hemophilia B, one for canavan's disease, one for ALS and one for limb girdle muscular dystrophy.

These trials are in various stages of clinical development and two of them, as denoted by the asterisks, have not actually been initiated at this time.

It is interesting to note that these are really all rare diseases and so AAV has proved to be a useful vector to try to approach rare diseases where again some of the target tissues are not actively dividing and, therefore, are not -- other vector systems are not effective in gene delivery.

(Slide.)

The second impetus for today's meeting is the recent emergence of data suggesting a possible association between AAV vector transfer and tumorigenesis. This question has arisen in the context of a particular mouse model of a disease called mucopolysaccharidosis VII or MPS VII. This is a lysosomal storage disease characterized by the deficiency of a particular enzyme, B-glucuronidase, GUSB.

In these studies AAV vector encoding human GUSB was administered to neonatal mice and the good news was that significant improvements in disease symptoms were seen out over a year. However, some of the mice developed hepatomas and angiosarcomas that were detected between eight and 18 months of age. And in light of these findings the investigator notified the FDA and we will be hearing from the investigator late this morning.

(Slide.)

So today's agenda topics include a review of the biology of AAV and

then a review discussion of the preclinical studies focusing on long-term data from animal studies using AAV vectors, the how, when, why of tumor development in mice, and a review of the natural history of B-glucuronidase deficiency in mice or the MPS VII mouse model. This afternoon we will focus on the data obtained from several clinical studies using AAV vectors.

(Slide.)

In terms of the format for today's agenda the focal point for all our discussions are going to be a set of symposium questions which should be in your meeting materials, and I urge you to get them out early and refer to them frequently. They were developed by NIH and FDA and in close collaboration, and they will guide the speaker presentations as well as the subsequent roundtable discussions.

To help guide the discussion of these issues and other salient points we have assembled a panel of experts who braved the weather conditions. We are unfortunately missing three people who may show up later today but they are coming from the hinterlands of the Northeast United States. But for those of you who made it, thank you very much.

Our co-chairs for the morning session will be Phil Johnson and Xandra Breakefield and in the afternoon session will be Phil Johnson and RAC Chair Claudia Mickelson.

(Slide.)

So our goals are not simply to exchange information strategies and perspectives but to address those particular questions and other salient issues and to develop a set of general principles and practical recommendations to guide the field.

(Slide.)

And many of you will recognize the next slide but I am always compelled to show to remind us of our over arching and common goals to ensure that the knowledge gained in each trial informs the current and future research and that we have a system in place that ensures that the safety of every single patient counts.

With that, I will close.

## SESSION I: ADENO-ASSOCIATED VIRUS (AAV): OVERVIEW OF BIOLOGY

## OVERVIEW OF AAV BIOLOGY

PHILIP JOHNSON, M.D.
OHIO STATE UNIVERSITY

DR. JOHNSON: We might need Terry to come up and run his computer for us.

Well, Amy just gave my talk so I can basically sit back down.

(Slide.)

The materials that have been distributed to you, I believe, are going to be very useful and I think we do want to try and stick to the questions that Amy and her advisors have put forward. I would admit to you that most of us are going to see the data for the first time today and I think it is important that we take time to digest that data and then discuss it as is intended in the roundtable discussion.

The purpose for me is to give an overview of AAV biology and that is somewhat of a joke. This is the only data slide that I am going to show you.

(Slide.)

On the Y axis is relative knowledge of AAV biology and on the X axis is P and O. P stands for me and O stands for other people that are sitting at this table like Nick and Jude. And the P value there is highly significant in relationship to

what their knowledge is versus what my knowledge is so it is with some trepidation that I tackle this particular topic. However, there are some things, I think, that we can all agree on.

(Slide.)

This is the difficulty with using a MacIntosh in an IBM world. My middle panel did not load there, which is basically a human figure, so all the arrows point to the right organ but this is really why we are here today. AAV now is being proposed for a number of clinical applications that are very important. Retinal disorders, arteriosclerosis, something I am very interested in as I age, hemophilia, which we will hear a lot about, other genetic defects, certainly MP VII and canavan's disease, arthritis. In the liver we are looking at delivering genes for again hemophilia in muscle. We are doing hemophilia and even vaccines. Cystic fibrosis obviously led the way for this particular vector and continues to lead the way in terms of clinical trials today. We have lots of data available there. And for the central nervous system and other neuromuscular disorders. Clearly this vector has tremendous potential.

(Slide.)

This is also a picture that many of us have shown over the years and this is again what I think started in many ways the surprise that Jude and others showed very early on that when you put a vector in muscle that you can get expression basically for the life of that mouse and so this has really driven a lot of why we are here today and why the vector has attracted so much attention.

(Slide.)

So the salient points that I want to cover in my very brief presentation because I want to get us back on time here, I want to say a few words about infection with wild type AAV, what we do and mostly do not know about that. I want to set the stage for Mark Kay and Terry Flotte because they are going to talk a little bit about integration and safety. And then I want to in a single slide try to compare for you wild type AAV versus vector. Are they the same? Are they different? And then finally say a word again in preparation for some of what I know Mark Kay is going to present, how do AAV vectors really work.

(Slide.)

Well, here is what I know about wild type AAV infection. That we have never been able to associate any disease with wild type infection and, in fact, serioepidemiologists have said that AAV, if you have been infected and have antibodies to certain serotypes, you might be protected from cancer, a particular type of cancer, cervical cancer. We have no idea why that is true. We could all hypothesize why it might be but, in fact, the data are there.

We also know that there is an age-related acquisition of antibodies and this makes perfect sense if you understand the natural history of this particular virus. It goes in nature with its helper virus, either adenovirus or herpes simplex.

It has been reported that there is a high seroprevalence to this particular virus and that makes sense again given the higher seroprevalence of helper viruses. However, the question really is are these antibodies neutralizing? Will they stop or prevent reinfection? We have done work in this area showing that relatively low numbers of people are carrying antibodies that neutralize 90 percent of a virus input.

There are multiple serotypes that infect primates, both human and nonhuman. One important distinction is that serotype 5 appears to be a sexually transmitted disease going along in nature with herpes simplex virus.

Clearly there are other adeno-associated viruses that infect other

species, and these have been relatively under served in terms of research.

So you put all this together and one could raise the question, and it certainly was raised many years ago, is a model vector for gene transfer.

(Slide.)

On the other hand there are a lot of questions we do not know the answer to. Does AAV persist in humans? Well, there certainly are data to suggest that it does but they are scant. They are not well documented and I think that a lot of work needs to be done. And if it does persist, at what sites? We know that the portal of entry is the respiratory tract for many serotypes but African green monkey kidney cells, primary African green monkey kidney cells can be induced to produce AAV. So it is in the kidney in monkeys. How does that happen?

And when it does persist, what is the copy number? We have really no idea what the copy number is of natural AAV persistence. Furthermore, we have no idea what the genomic form of the virus is in our bodies sitting here in this room today and what the fate of those forms is over time. We really do not understand any of these questions. Does replication occur? Does rescue occur in the natural setting?

And finally are there any consequences? Certainly the long epidemiologic history would suggest that there are none, that we have not been able to associate disease and certainly not been able to associate cancer with the wild type virus.

And really I think in the late '60s and early '70s nobody really cared about this virus anymore. It sort of disappeared from most people's agendas except those small group of people that have been interested in this over the years as a model for molecular biology and DNA replication.

(Slide.)

One of the things that was denoted early on by Dave Hogan and others was that this virus actually persists in cultured cells and notice I am using cultured cells again as the model because that is really where all the data come from. And then in the early '90s Cotton, Jude and others identified that this virus actually integrates in a site specific location on human chromosome 19. And the locus was designated S1. It is on the long arm of chromosome 19.

Several things are pertinent, I think. For this integration event to occur, rep is required, so therefore wild type virus has rep. There is a requirement for the terminal repeats of this virus, a complex, compact structure. There is obviously a requirement for the S1 locus.

But the questions that remain open are what is the mechanism? How does this actually occur? I think there are some very nice models put forth by Lyndon Burns, his group and others that can speak to this but I am not sure that it has really been well documented.

What is the frequency of integration? This is another common misconception, I believe, that the virus always goes to chromosome 19. In fact, it does not. It goes other places. Roughly 75 percent of the events in cell culture will end up at chromosome 19 but there are 25 percent of integration events at other places so we know that it does not always go to chromosome 19. It is site preferred and site specific but not certainly exclusively chromosome 19.

(Slide.)

So that is -- everything I have just said really has a lot to do with wild type. Now let's compare it with recombinant vectors that have become popular.

(Slide.)

This is a diagram just to let you know what the difference are in the genetic structure. Basically wild type virus is composed of the terminal repeats in the two genes that were often with rep and cap. On the other hand, the vector does not have rep. It does not have cap. It only has the terminal repeats and hence the recombinant vector really only contains about 300 nucleotides of AAV and no genes. No gene products.

(Slide.)

So if we ask a series of very simple questions to compare we come to the conclusion that vector and wild type virus are actually very different. If we look at the portal of entry for the wild type virus, we believe that it is respiratory tract and the genital tract. It makes sense again following in nature the helper viruses. However, vector, there are various routes of entry depending on your particular purpose. Is it intravenous? Is it aerosolized into the lung? Is it injected intrahepatically? So there are a variety of portals of entry that are not found in nature when we use the vector.

What is the dose? Well, obviously with the vector it is going to depend on the application but it is likely to be high relative to the inoculum that one would receive in nature. However, remember that the wild type virus can replicate in the presence of the helper virus and, therefore, the titers might be fairly considerable as the virus is replicating, however, that would diminish rapidly as the helper virus is eliminated using immune mechanisms of the body.

I have already spoken about persistence of the wild type virus. We really do not understand that. We know that a vector does persist. Multiple labs, multiple publications now show that this vector can persist for long, long periods of time.

We know that the wild type virus has rep but do we know that the vector does not have rep? Do all vector preparations lack rep? Well, the answer is probably no. Probably some of these vector preparations do include rep and have rep containing -- rep gene containing particles within them. What is the effect of that and how much does it influence integration, nonintegration and persistence?

Finally, we have to worry about transgene effect and obviously with wild type there is no transgene so there can be no transgene effect but with the vector each virus might well be different because of the transgene. So this slide really does point out that the vector is different from wild type and I think that is important to keep in our minds as we move forward today.

(Slide.)

Is it fair to extrapolate from wild type what we know about wild type? Should we learn more about wild type? And I think the answer to that is sort of, maybe and it depends because it really is going to depend on what application you are looking for, whether you contain rep or not, and what we think we can learn from the biology of the wild type virus itself.

(Slide.)

In the last few slides I want to make a few comments about how vectors might or might not work.

(Slide.)

This is the way that I view recombinant AAV vectors today. I really think they are single strand DNA delivery vehicles that are unique. It is basically a protein, vp3 wrapped around a single stranded DNA. This virus attaches to the cell via a cellular receptor, whatever that happens to be for AAV serotype 2. Certainly at

least part of the interaction occurs through heparin molecules.

The virus attaches to the cell surface and then penetrates to the nucleus where it uncoats and dumps a single stranded DNA and there -- that is when the fun begins because we really do not understand very much about how the single stranded DNA becomes double stranded and then what forms in the cell are responsible for both transduction, that is the expression of a transgene protein. We also do not understand how it persists or why it persists and I think we will get at some of that with the presentations this morning.

(Slide.)

Clearly a number of people have done these types of experiments to show that the vector genomes persist. They begin on the left as single stranded low molecular weight molecules and they migrate over time to high molecular weight molecules that are most certainly concatameric.

(Slide.)

The real question is are these integrated high molecular weight concatamers or are they episomal high molecular weight concatamers? And I think a number of laboratories are investigating this rigorously now and certainly in our hands in muscle the majority of the genomes that are persisting long-term are episomal and not integrated. I will not show data today. I think Mark is going to address a lot of that.

(Slide.)

So, in summary, wild type AAV, no disease or tumors that we are aware of in the world's literature. The vector -- we also to my knowledge have not seen any disease or tumors up until the recent report from Sands' laboratory. It is important to remember that vector and wild type are different, however, and that we have to keep that in mind. Integration of the vector, I think, is a very open issue and is an evolving topic.

But the most important thing that I think we should have happen today is that the data should drive our decisions. We should really look hard at the data. If we do not have the data, we should generate it and then we should make decisions really based on the data that we have in front of us.

So, with that, I want to turn to Mark and have him begin his presentation on integration.

## INTEGRATION OF AAV INTO HOST GENOME

MARK KAY, M.D., Ph.D.
STANFORD UNIVERSITY SCHOOL OF MEDICINE

DR. KAY: I want to thank you for inviting me to share our data and to discuss with you what we believe are some of the important points in AAV transduction in vivo and specifically address some issues about integration of AAV genomes in vivo.

(Slide.)

Can you focus that and maybe turn the lights down?

This actually represents an old slide of a collaborative study we did with Richard Snyder and colleagues from his group and my group back in 1996. It shows what happens when you take an AAV vector and inject it into the portal vein and look at gene expression in mice. And each of these lines represents individual mice that got a dose of an AAV Human Factor XI construct.

There are two things that I want to point out here. The first is that gene expression is therapeutic. This level of Factor IX would be curative. And that it

persists long-term for the life of the animal. Secondly, when these studies were done, for all intents and purposes from the acute toxicity point of view, it appeared to be an extremely safe vector.

What really intrigued us, and unlike what we have seen with other vector systems, was the slow rise in gene expression that occurs over about three to six weeks. So over this period of three to six weeks what we find is that we have a slow rise before hitting this steady state level of gene expression and since that period of time we have been interested in studying what the molecular events are once the vector genome gets into cells to reach this state.

(Slide.)

Now what I decided to do was to try to summarize certain points that I think most people will accept and then briefly summarize some published data and then spend a little more time talking about data that is not published. The rise in AAV mediated transgene expression occurs over this three to six week period before reaching the steady state. Now during this time there is a concordant disappearance of the single stranded AAV genomes and the appearance of double stranded genomes again over the same period.

The molecular structure of the double stranded genomes includes monomer circles and linear forms, small and large concatamers, and if you isolate the large concatamers away from the monomers, we have found that the concatamers are pretty equal in head to tail, tail to tail and head to head structures. I will come back to this later.

(Slide.)

Now the other thing that has been somewhat of interest is that if you inject the routine doses that most labs inject into the portal vein and you look one day after gene transfer, if you do DNA in situ hybridization, almost every single hepatocyte nuclei contains AAV genomes. Now if you take these cells from the liver one day after injection and culture them and then add adenovirus to basically allow the single stranded genomes to become double stranded genomes quickly, we find that again almost all of the hepatocytes expressed from the AAV genome. Not only do we believe that we are getting uptake into the nuclei in almost all hepatocytes but the genomes are potentially biologically active.

What is somewhat of a puzzle is even within a reasonable dose range that no matter what you do you can only get a stable transduction in about five percent of the hepatocytes. So even though all the cells take it up, you get it in the nucleus, only five percent become stably transduced. This has been determined by looking at transgene protein detection. That is if you use intracellular markers or markers where you can use immunohistochemistry, RNA in situ hybridization looking for message RNA production, and finally doing DNA FISH analysis. The correlation between this is really nice.

Now when I say around five percent, that can vary between two and eight percent but it is approximately five percent.

Now this brings an important issue up. Why is it only five percent? Is there a changing subpopulation of hepatocytes permissive to transduction? This is somewhat controversial. This is our hypothesis at the time. We have some data to support this, although it is clearly in my mind not definitive.

What we do know and as Phil pointed out, transduction is not associated with cell cycling. There are several lines of evidence to support this. You can do partial hepatectomies and inject AAV during rapid periods of liver

regeneration. There is no increase in transduction and more importantly if you do BRDU labeling and look for cotransduced cells with BRDU there is no association. This is very different than what we see with retroviral vectors. So again cell cycling does not appear to be an important process at least in liver in vivo for transduction.

(Slide.)

Now the question comes in, is how do these single stranded genomes uncoat and then become double stranded genomes? This is a question that Phil raised and this is something we have been interested in. Now again some of this data is published. There are two reasonable possibilities. One is that you get second strand synthesis and this has been, I think, really well worked out in many of the cases for the wild type virus but in the vector it is not always so clear whether this can occur versus annealing of the plus and minus strands to form a double strand.

(Slide.)

As I am going to show you three lines of evidence, we highly favor this process.

The first set of experiments that we did to address this, and a lot of this work, I should point out, was done by a post-doc in the lab, Hiroyki Nikei, was to make AAV genomes that were actually methylated adenine residues at a specific sequence.

Now adenine residues that are methylated -- in mammalian cells there is really no way to methylate adenine residues or to demethylate adenine residues. This is something that is fairly unique to prokaryotic cells. So the idea was that we would methylate adenine residues and that we would follow the methylation patterns in the mice as the DNA became double stranded and as you got stable gene expression.

So we would inject the mice with this virus and the virus expressed just as well as the nonmethylated counterparts, and then we used a number of different restriction enzymes to ask the question are the AAV genomes methylated. What we found by and large is that the majority of the genomes remain double strand methylated.

If second strand synthesis were occurring, we would either see hemimethylated or unmethylated genomes and we do not see evidence for that.

(Slide.)

A second line of evidence, although this is less conclusive, was to make vectors that are basically identical in sequence except for eight base pairs that either knockout a Bam HI or an Eco R1 site.

What you can do then is you can mix these two together and then look in vivo at what kind of structures you find.

(Slide.)

If there were -- if a second strand synthesis and then rolling circle or some sort of nonrandom linkage occurred, you would expect to see these homoconcatamers when you cut with either a Bam H1 or Eco R1, and this is not what you see. What you see basically is a ladder when you cut with either Bam H1 or Eco R1, which suggests random linkage.

What is more important from the standpoint of annealing is that if you look at these genomes there is evidence for mismatches and, secondly, there is evidence for mismatch repair. Again suggestive of annealing and again this data has recently been published so I do not want to go into all the primary data.

(Slide.)

Now the third piece of evidence which is new in our lab comes from the fact that even though we have not been able to demonstrate second strand synthesis does not mean that it could not occur under some conditions. So what we decided to do is to try to do an experiment where we could isolate the minus strand genomes from the plus strand genomes. Now this has been extremely difficult to do in the virus because there is not really a good way to do this in bulk to get a pure population of plus and minus strands, full-length genomes.

So what we hypothesized is that if second strand synthesis did occur -- I am sorry, if you injected just a minus or a plus strand and second strand synthesis was allowed to occur, you could get double strand synthesis. If annealing was the mechanism then if you injected one strand versus the other you would get no -- you would get no double strand formation. But if you injected both together or separately you would.

(Slide.)

And to do this experiment, Xing Chin in the lab developed some DNA plasmid vectors that are depicted here. They have the AAV ITRs and they have a Factor IX expression cassette. What we can do with this plasmid is we can take it through single stranded bacteria phage and we designed these just so that under conditions -- certain conditions what happens is you get single strand molecules out that contain your expression cassette but also have this bacterial backbone in some of the phage sequences.

We really do not want this in the experiment so under the right conditions you will get annealing of these two and what you can do is cut with either an enzyme that releases the expression cassette with or without the AAV ITRs. If you release it with the AAV ITRs you are basically recapitulating an AAV genome and by reversing the order of the F1 in the bacteria phage you can make a pure population of plus strands and a pure population of minus strands.

(Slide.)

Then what you can do is you can inject these into mice. Now what happened was if you take plus or minus strand alone and you injected the tail of a mouse under conditions that is known as hydrodynamic transfection procedures, you can get DNA in at least 40 percent of the mouse and this was developed by Dexy Lu and Jon Woolf's group.

Now if we take single stranded DNA, either the plus or the minus strand alone, we see absolutely no gene expression over time. If we take a double stranded circular plasmid, you see very robust expression early on within one day but then that falls off because that is what we see with double stranded circular plasmids.

But if we take either the plus strand and the minus strand and mix them together on ice just before injection or if we take the DNA and inject it separately into two different tail veins, that is one tail vein that is plus strand and one tail vein that is minus strand, you see this slow rise in gene expression that stabilizes just after three weeks. This is very similar to the kinetics that we observe with AAV.

What is even more interesting, at least to me, is that if you take away the AAV ITRs you see the same expression pattern. So again this is not consistent with second strand synthesis as the mechanism and that annealing is probably playing an important role <u>in vivo</u> towards the stable transduction state.

(Slide.)

And just to show you that what we have done is we have analyzed molecularly these single stranded genomes that get both strands and we find equal

mixtures of head to tail, tail to tail and head to head concatamers similar to what we have seen with AAV.

(Slide.)

So what we think with the mechanism of AAV transduction is that there is annealing of complimentary single stranded DNAs and this results in double strand DNA formation. There is random linkage that results in concatamer formation.

The evidence for this, again to summarize, is the persistence of the double stranded adenine methylated vector genomes, the presence of double stranded heteroduplex genomes when the two vectors with small nucleotide polymorphisms are coadministered, demonstration of mismatch repair, absence of transduction using plus or minus genomes alone, and the equal abundance of head to head, head to tail and tail to tail concatamers when you isolate the low molecular weight away from the high molecular weight concatamers.

(Slide.)

Now what about integration? What is the published literature that would support integration in vivo? Really I would say that most of the data out there would support that there is some level of integration in the liver and although there is some indications in other tissues such as muscle, I would say in my opinion there has not been at least published definitive data to support that.

(Slide.)

Carol Miao in our lab did pulse gel electrophoresis and showed that the AAV genome co-migrated in the multimegabase range right where the mouse chromosomal DNA migrated. She did DNA FISH signals and showed sister chromatid metaphase signals, again indicative of integration. And probably most convincingly here, Hiroyki Nikei actually made a vector with bacterial origins of replication and then was able to inject these AAV vectors and then clone out DNA plasmids after cutting the genomic DNA and then passing these through bacteria. And then what you do is you basically trap AAV juncture integration sites. So you are actually covalently linking the AAV to the genomic sequences where integration occurred.

Eighteen molecularly characterized in detail and four of these integrations were found in known sequences. One was in the entron of mouse alpha 1 collagen gene in the 28 sRNA transcribed region. Another protein with some pluride channel and some other protein with some homology to this enzyme.

More recently, Jim Wilson's group has used a selection procedure, which I am going to talk about a little bit more in a minute. But basically the idea is that you use genetic models where only hepatocytes that are genetically corrected with an integration event supplying back a missing gene product will survive. And they have a huge selective advantage and they can repopulate the organ. Again, this is also evidence for integration.

(Slide.)

So what do we think is going on at this point? We have the two genomes coming in. We believe annealing is a process. It is our hypothesis, and not proven at this time, but we hypothesis that annealing may be one of the rate limiting steps. There may be others.

Why it takes a period of time to get stable gene expression? You get these circular monomers, which are pretty predominant in the liver, and I think also in muscle. I think Terry is going to mention this. You can get concatamerization in the dimers and then larger concatamers. There are probably some episomal linear

concatamers and then we know that there are some integrated genomes and whether they are concatamers or single is something I will address in a second.

The point is all of these studies to date do not really address the exact pathways of this and do not demonstrate what proportion of the DNA is integrated versus episomal.

(Slide.)

So what I would like to do now is try to tell you some of the ways that we have tried to quantitate this and determine the amount of integrated DNA.

What we have done is we are using a technique known as partial hepatectomy. If you take out two-thirds of the mouse or the rat liver, almost all of the cells will divide once or twice to restore the original hepatocyte number.

So what we do is we transduce mouse liver with AAV and we also can use controls with integrating and nonintegrating vectors. And at different time points we do a partial hepatectomy and then ask the question how much gene expression is left. We can measure the transgene product and the vector genomes before and after partial hepatectomy. The idea being with cell division you will lose episomal forms but you will retain the integrated forms.

This is the type of study that we have done.

(Slide.)

This is a proof of principle that this type of approach will work. In this experiment what we have done is we have used a known integrating vector that happens to be a transposon and this is a control for that in which the DNA -- the same DNA is injected without the transposon. And then what you do is you wait out almost a year. You do a partial hepatectomy. In an integrated situation, gene expressions will store to the pre-partial hepatectomy level. And when you do the partial hepatectomy with a nonintegrating vector, you get a log loss or so in gene expression.

(Slide.)

So this is the type of experiment that we did with AAV. So these mice were given AAV at time zero. We waited about,in this experiment,12 weeks and then half of the mice had a partial hepatectomy and half of the mice did not. And you see here there is almost a tenfold drop in gene expression with partial hepatectomy. This suggests that a lot of the expressing genomes from AAV in the liver are not integrated in episomal.

(Slide.)

What about the DNA quantity? This shows you the raw data. This is a concentration curve of copies per cell. This shows you in individual mice prehepatectomy the AAV band. This is double stranded DNA. And post-hepatectomy and other -- what you see is you see a huge drop off. These are the animals that did not get hepatectomy.

So what you can see here is there is a big difference. We have quantitated this and what you can see is prehepatectomy. In this experiment there is about one copy per cell. Post-hepatectomy it falls dramatically. And with no hepatectomy again it is indistinguishable from prehepatectomy.

So there is about a tenfold drop in gene expression and a tenfold or so drop in DNA genomes. In fact, we have quantitated this even further.

(Slide.)

In most --

(Slide.)

Let me just show you that if you wait one year after giving AAV and then you do a partial hepatectomy, these are no hepatectomy controls, these are partial hepatectomy, you see a similar drop in gene expression. What this suggests is that over time there is not a predominance. That is there does not appear to be a big change in the proportion of episomes versus integration so if integration events do occur over time there is not a substantial amount of that occurring.

(Slide.)

Let me say that what this means at this point is that the amount of gene expression and the amount of genomes that are present in the animals correlate so that means that the loss of genomes, that is gene expression appears to parallel the amount of episomal versus integrated copies.

Now with that said, I just want to say in a more recent experiment we have done -- we had one anomalous animal that had over 50 percent integrated genomes and we do not understand why that animal is different than the other ones. In these experiments there is a total of about 12 or 15 animals that have been given partial hepatectomy.

(Slide.)

Now the thing about this is this tells you the proportion of genomes that are integrated versus not integrated but it does not tell you the proportion of transduced cells that actually have integrated copies and I think this is important.

This is depicting an experiment. Some of which -- this experiment was published by Jim Wilson's group but I am going to show you we are taking a slightly different approach to try to get more quantitative data.

What we are using here is we are using a mouse system that is called tyrosinemia Type 1. These mice have a defect in an enzyme called FAH. These hepatocytes have a cell autonomous lethal effect and it is due to the build up of tyrosine metabolites, and it affects only cells that have the mutation. If you give these animals no therapy they have chronic hepatocyte regeneration and turnover and the animals eventually die of liver failure. There is a human disease that is very similar to this in phenotype but it is rare.

Now what Marcus Grompy's group has shown is that you can transplate genetically normal hepatocytes back into these animals and because these cells have such a selective advantage you can inject less than 1,000 cells and repopulate the liver of all these animals. And you can actually isolate these animals before repopulation is finished and get nodules out and then look at these molecularly.

Now Jim Wilson injected high doses of AAV into these type of mice, repopulated and looked at nodules and he analyzed -- now what you do is you lose all the episomal forms but you maintain integrated forms with multiple cell divisions, and what he did was he showed that the copy number was about one to two. So the integrated AAV appears to be either single or double on average.

Now there is also a drug called NTBC that basically short circuits the metabolic defect and these mice can be relatively normal. This is important because this is the type of experiment that we have started to do to try to really quantitate the number of cells that have integrated genomes.

You take the mouse -- you keep them on NTBC so their livers are normal and not regenerating. You infuse them with this dose of an AAV vector that has this enzyme, wait about six weeks, and then you isolate the hepatocytes. So these are animals that are transduced. There is no selection for integrated copies because they remain on this drug therapy. And then you transplant a million of these cells into

the naive FAH deficient mice that are off NTBC.

Now episomal and non-AAV hepatocytes are going to be lost. Okay. But what you will -- is you will select for integrated AAV genomes containing hepatocytes and you will get clonal repopulation. Now we are injecting a million cells and we know based on historical data that about five percent or about $5X10^4$ should be genetically modified with AAV. If 1,000 cells have integrated then the animal should survive based on what I have already told you from published literature.

So if we assume that 1,000 is the minimum number,and it actually may be lower than this, and we are saying that we are injecting 50,000 total transduced cells, we could argue that less than a few percent of the actual cells that were initially transduced contained integrated genomes.

Now this exact number could probably fluctuate because additional studies need to be done and better controls. This is very preliminary. What it says is that the number of cells -- the amount of episomal versus integrated DNA correlates with gene expression and it also probably represents the number of cells that have integrated events.

(Slide.)

So this is a summary. Five percent of the hepatocytes were stably modified with AAV. The proportion of integrated genomes is small. Generally less than 10 percent of double stranded vector DNA in these cells. Again, I think the partial hepatectomy method probably will over estimate integrated copies.

Gene expression from integrated and episomal AAV genomes parallels the proportion of vector DNA in each state. There is no detectable increase in the proportion of integrated genomes over time. The proportion of transduced cells with integrated genomes is small. Most integrants based on Jim Wilson's data are one or two copy genomes and we are in the process of repeating those studies for confirmation as well.

(Slide.)

I would just like to say that a lot of this work was done by Hiroyki Nikei, a post-doc in the lab; Carol Maio; some of the early study that I mentioned was done with Richard Snyder; and a number of talented technicians in the lab. So I will stop there for this part of the talk.

DR. JOHNSON: Thanks, Mark.

We have time for about two questions if the audience has one or two burning questions if we could have the lights up for just a second.

Anybody have a question for Mark on this portion of his talk? He is going to move on to talk about some safety data.

Yes, Rob?

DR. _____: Nice presentation, Mark. I have a question about the organization of the episomal DNA and the integrated DNA. You mentioned you found all molecular confirmations which is predicted by the physical association of the genome. Earlier, though, most labs were reporting only head to tail concatamers, which would predicted from an alternative DNA synthesis model. Do you think there is some selection for one form versus the other?

DR. KAY: I think that is an excellent question and I am glad you raised that point. We actually published and hypothesized as well that most of the concatamers were head to tail in some of our Nature Genetics papers.

What we have found, however, is that if you actually isolate the circular monomers, and there is quite a bit of those, away from the very high

molecular weight concatamers, that is if you physically separate those in agarose gels and you can take and extract that DNA that only represents the high molecular weight concatamers and then you do restriction digestion, you see equal numbers of head to head, head to tail and tail to tail.

So what we believe is that we have misinterpreted our previous data because of the contamination of circular monomers and also other small molecular weight forms but when you isolate those away, everything becomes consistent.

Does that answer?

DR. _____: (Not at microphone.) Mostly. The other part of the question is the integrated forms in vivo with only one or two exceptions have always been head to tail. That seems to be --

DR. KAY: I think that there is a very small paucity of data on integrated forms in vivo with vector and I think right now the only data that I am aware of is Jim Wilson's data and I am not sure -- I do not remember if they have characterized it that carefully but I think there is going to be a number of other studies that we are doing and I think others are doing that will look at that more carefully.

The question is which form actually gets integrated and what is the mechanism of integration? I think that that is unknown but one thing I would say is I do not think that it is dependent on the AAV ITRs because if you take that single strand experiment and you lop off the AAV ITRs you see the same formation of the concatamers, with or without the AAV ITRs, the same kinetics of expression. So whatever the mechanism is, in my opinion, you do not need the AAV ITRs.

DR. SLY: You emphasized that most of them do not integrate but the other way to look at it is that maybe 10 percent do.

DR. KAY: It is probably less than 10 percent in most cases. It is definitely detectable. I would say it is between -- my guess is it would be between one percent and 10 percent.

DR. SLY: (Not at microphone.) Appreciable amount.

DR. KAY: Yes. But if you compare that to retroviruses, which, you know, people have worked on a long time, it is actually a low number.

DR. JOHNSON: Please, we would ask the questioners to identify themselves and their organization before they speak.

DR. KAY: Yes.

DR. JOHNSON: We will have this question and then one more from Dr. Samulski.

DR. _____: In your recombination studies do you have any idea with your hepatocytes that have integrated genomes where their growth characteristics are altered, and I guess there could be a bias if one actually had some impairment in mitosis that you would not get repopulation. So whether you have an integrated --

DR. KAY: I am sorry. I did not hear.

DR. _____: The question is whether you have an integrated genome, whether it disturbs the actual cell cycle and the mitotic potential of that hepatocyte which could potentially bias some of the interpretation, I guess, if you had some impairment of growth in cells and the AAV genome integrated.

DR. KAY: I think that is a good point. I mean, I cannot answer that definitively. What I can say is that in the FAH deficient mice relative to retroviruses the same sort of rescue is seen with AAV. And when I have talked to Marcus about this, he has seen no evidence that there is any type of unusual business. If you give a

good enough dose you can repopulate the animal with the same type of kinetics that you can with retroviruses. Although again these studies are very preliminary and we need to do more studies. I mean, I think this is something that we are keeping in the back of our mind.

What I can is at this time there is no evidence to suggest that but there is no definitive evidence to support the other either.

DR. JOHNSON: Jude, one final question.

DR. SAMULSKI: Mark, I want to make a comment and then ask you a question. The comment is I think we need to be careful in taking plasmid DNAs and introducing them into animals and observing observations and then concluding this is how the virus probably carries out these steps maturation to a molecular fate because typically I do not think it would be fair to take lipids and put in materials and then say this is how retroviruses deliver their genomes because it is basically a lipid and we are getting it into the cell and so forth. So that is just a general comment that I do not want get misperceptions out there that we are mimicking what the virus is doing because we do not understand a lot of those steps.

The question is in the animal where you said you saw over 50 percent integration events, do you have any inclination on why that type of event could occur? It suggested it could happen and I guess the critical question is was there something unique to that vector prep or animals that might have gave a propensity for a high level of integration?

DR. KAY: The first thing -- let me make a comment about your first comment. I would agree with what you say but I am trying to emphasize after the genome is in the nucleus and I know that still is different because it is not complex to the viral proteins. I am trying to use it as one piece of evidence with the other two pieces of evidence about the annealing but I do accept the point and I think it is important because prior to getting into the nucleus and even once it is in the nucleus a lot of other events have to take place.

The question about what was specific about that animal, I have absolutely no idea. The livers appear normal. With that particular dose and batch of vector, three animals were administered AAV. The dose was higher than what I showed but only one of three animals had that high of an integration so I have no idea.

## SESSION II: PRECLINICAL STUDIES USING AAV VECTORS

DR. JOHNSON: Okay. We are going to ask Mark to move on to the second presentation which is on long-term safety data.

## LONG-TERM SAFETY DATA

MARK KAY, M.D., Ph.D.
STANFORD UNIVERSITY SCHOOL OF MEDICINE

(Slide.)

DR. KAY: So obviously since the observation that AAV appeared safe and therapeutic with Factor IX, we have been very interested in pursuing clinical trials. And, as I am sure you are all aware, we have worked with Avigen and Kathy High and, as a group, we have moved forward in the clinic, and you will hear more about that later from Bert Glader.

But what I would like to say is that toxicity can be divided into different issues and really what we want to focus on is long-term toxicity and we have done quite extensive studies of acute toxicity in mice where probably thousands have been injected. Lots of rats, some dogs. We have done biodistribution, germ line

transmission, and I am just going to summarize in two slides.

(Slide.)

Probably the largest acute toxicity study we did was in collaboration with Avigen done under GLP conditions in which we started with a large number of rats and gave them either the excipient, an AAV null vector or an AAV Factor IX vector that we want to use in a liver based trial at different doses. And then we sacrificed the animals. We measured lots of things, including hematological parameters, serum chemistry, histopathology, cytokines, and then did biodistribution studies.

(Slide.)

I will just say that, you know, these acute toxicity studies have basically turned up nothing in regards to safety issues of the vector.

(Slide.)

We have also done a gonadal distribution study in dogs. We use a null vector that has a truncated beta gal expression cassette so it cannot express any gene product. We inject this in the dogs. Doses range from $3\text{-}7.5X10^{12}$ particles per kilogram and we do semen collection at different time points afterwards.

(Slide.)

I just want to show you that we can inject into the hepatic artery using a balloon catheter that goes in by standard clinical technologies the invasive radiologists do. The catheter goes in, the balloon is filled, and you can inject dye to prove that you are in the hepatic vasculature. You wash out the dye, let the dye wash out, and inject the vector.

(Slide.)

I am showing you a Southern blot from one of these animals because I think there is one important long-term safety issue here. If you do a concentration curve shown here, you take liver sections from different pieces of the liver, the different lobes, lobes one through four, you can do Southern blots and also look at the spleen. We see no DNA in the spleen at a pretty low sensitivity and we have summarized all this data here in the three different dogs that we have injected in the different lobes.

(Slide.)

There is a little bit of variation between lobes but it is fairly concordant. We have also seen this in mouse and rat. Importantly, there is no vector genomes detectable by Southern blot at this level of sensitivity in these animals.

(Slide.)

So just to summarize a lot of data in dogs, rats and mice, we have administered doses of AAV that are 50-fold higher than our proposed starting dose that we want to do in patients. There has been no evidence of serum toxicity measured by chemistries, liver functions, hematologic parameters, cytokine levels, and absence of germ line transmission. Again studies done in collaboration with Kathy High's group, less than one AAV per 3,000 genomes, and histopathology has been relatively unremarkable.

(Slide.)

Now what I have done is I decided to try to summarize as much long-term safety data as I could and I would like to acknowledge that there were a number of investigators who shared data with us. I guess I can cross me off but Kathy's group, Gordon Watson's group at Oakland Children's, Kathy Ponder at Wash. U., a group in France, and Inder Verma's group.

So what I would like to do is talk to you and show you some long-term safety data injecting AAV in different animals. Now when I do talk about hemophilia B dog studies, I realize that Tim Nichols at University of North Carolina has been involved with all of these groups and a collaborator, and that even though I have only listed single names that there are a lot of people involved with these studies but for simplicity I have listed the principal investigator.

(Slide.)

Now the first thing I want to do is talk about data from our group and to show you that -- and this slide, the first of three slides -- we injected in this slide 18 mice with various AAV constructs into the liver by one method or another. Either direct liver injection or portal vein injection. And the dose here is total dose per animal. If you want dose per kilo you should multiply by about 50. This is vector genomes. Portal vein, $3X10^{11}$ to about $1X10^{11}$ in direct liver. Different strains of mice, NUDE mice, Black-6, et cetera. They were all around five to eight weeks of age at the time of injection.

And these animals that I am listing from our lab are all animals that were followed for one year or longer and their livers were examined at some level. Most of these animals had their livers removed for molecular analyses and what we do is we cut the livers up into little pieces and aliquot them out to different studies.

We can see in this slide that they varied from 12 months all the way to 19 months in follow-up. None of these animals had any liver tumors.

(Slide.)

A second slide shows a continuation of that. Again different vectors, different -- these were all portal vein injections. $2.4X10^{11}$ to $3X10^{11}$. These animals were followed for 12.5 and 14.5 months as well.

I should also point out that in this slide, many of these animals all the way up to here used an old prep of virus that actually was contaminated with wild type AAV so the issue about wild type contamination, at least in this group of mice did not have any liver tumors.

(Slide.)

Now we have also done dog studies. As I have mentioned, we did these null dogs but these were followed for short periods of time and sacrificed after about five months. We have two dogs that we have published on. Again a collaborative study with Tim Nichols and Richard Snyder. Some of these animals are out over -- one animal died at 795 days. I should point out this is the dose they got per kilo in the portal vein. The animals were about three months of age.

We followed this animal for 795 days. When it developed a spinal and brain hemorrhage, we sacrificed and the gross pathology, including examination of the liver was normal.

We have another dog that has still survived. It is still making about one percent canine Factor IX. It had an abdominal ultrasound in January and even after about 1,100 days there is no evidence of liver tumors.

I just want to point out that one of these -- for the null dog studies we had to use very old dogs because we needed to get semen samples and these dogs were well over seven years of age. We do not have the exact age. One of the animals did have a tumor but again it was found five months after injection of AAV and this tumor is not uncommon in older dogs.

(Slide.)

This is a summary of Kathy Ponder's data at -- so, in total, we injected

29 mice and we have data from two long-term dogs and three acute dogs.

Kathy Ponder's data is depicted here. She has followed animals for 300 days to 1.2 years. She has given some very young animals vector by intramuscular or intravenous injections. Again different animals, different ages, different doses. And in none of her animals did they find any evidence of liver tumor.

(Slide.)

This is Gordon Watson's data and I brought his dataset, although it is small, there is four animals. These were actually the MPS VII knockout mice that we will hear more about later. They were injected again in the neonatal period either by IV or some IV and intrathecal injection with doses of an AAV-C and B beta GUS vector. And again these animals were all followed for over a year and there was no evidence of liver tumors in these animals.

(Slide.)

This is Inder Verma's data. These four animals were published in Molecular Therapy. This is a follow-up.

This first dog has been followed out almost two years. Had a normal liver ultrasound in January of 2001. Again these animals were three to 12 months when injected.

This animal died seven months after therapy. No gross abnormalities were found.

This animal was followed a little over two years. Again normal liver ultrasound.

And this animal was just under two years and also had a normal liver ultrasound.

(Slide.)

This is Kathy High's data and what I am showing you is her hemophilia B dog studies with IM injection of AAV, CMV canine Factor IX. These are the ages again of injection. These are the doses. Some of these animals have been followed out over three-and-a-half years. Many of them two years. The minimum period of time is 17 months.

These animals have also had muscle as well -- many of them have had abdominal ultrasounds as well, even though they got this vector by IM, and there is no evidence of tumors.

(Slide.)

This is a continuation of her data. Some of these animals down here -- there were four animals that received either portal vein or mesenteric veins to liver directed therapy, followed for five months to 12 months.

This animal is alive four months after therapy, normal liver ultrasound.

Followed ten months, had a liver biopsy, normal.

Followed seven months, died, did not have any evidence of tumor.

And followed six-and-a-half months, alive and still being followed, and no evidence of tumor at this time.

(Slide.)

This is the French group. You cannot see it well but these are monkeys and these monkeys have all been given intramuscular injection of AAV. They varied in age at the time of injection from years to months. They have been followed eight to 18 months and they are alive, and they have had various procedures, et cetera, but there has been no evidence of tumors, et cetera.

(Slide.)

So if we summarize the data that I have presented to you, since we used a cutoff of 12 months, this number might -- may be a little bit higher but we had 56 liver treated mice followed between 10 and 19 months without evidence of liver tumors in any of these animals.

Fourteen liver treated dogs, 11 with hemophilia B and three normal followed for more than four months to more than three years.

Eleven muscle treated hemophilia B dogs followed for four months to more than three years.

Eight muscle treated nonhuman primates followed for eight to 17 months, and again there is no evidence of tumors or long-term problems in these animals.

(Slide.)

Now what I would like to do is in the last couple of minutes talk about an experiment we have done in collaboration with Mark Sands. You are going to hear more about this MPS VII mouse in a few minutes but Mark graciously shared some of his liver tumor samples with our lab and I want to extend thanks to Hiroyki Nikei who has worked extremely hard over the last few weeks to generate the analysis that I am going to show you.

So basically what we did is we isolated DNA from three of the tumors that he sent us.

(Slide.)

And this is a depiction of the map of the AAV vector that he will talk more about that we injected into these animals. We used in this experiment a 2 Kpn and Eco R1 probe. This particular Southern represents a Hind III digest. This Hind III digest cuts outside the vector. If there is an integration event you would expect to see unique bands at different sizes because again it does not cut within the vector.

(Slide.)

This is a control here. Hind III. Again this is a plasmid control added to naive liver DNA showing sensitivity down to less than .1 copies per cell. And actually with over exposure you can get to .03.

(Slide.)

This is a control cell line, a positive control. This is a retrovirally transduced cell line. It has a different restriction map but it has an endogenous GUS gene in it and this is the same line without the retrovirus.

These are the three tumor samples. There is no evidence for AAV genomes in this particular blot.

(Slide.)

We did a second digest with a Kpn enzyme. This cuts once in the vector. This should -- if there is a single integration event you should get a single band at different sizes in different tumors. If there is concatamers what you would see is a single band unit length or depending on which way the concatamer was. This is a one copy standard positive control retroviral line and this is our livers from two of the three animals. We had a third one in a second blot but this blot looked nicer, and again they are negative in all three examples.

(Slide.)

This is a Kpn Hind III digest. Again this is a one cutter and a no cutter. The idea being if we saw a single integration event we would change the size of the band by doing this double digest. Again we do not see any evidence for any AAV genomes at pretty high sensitivity.

(Slide.)

Now this is where we get confusing and I do not have explanation for this result but it has been repeated with the same result.

This is a Bgl II digest and the idea here was to cut something internally that would actually give you a copy number if we did see some evidence for integration.

And what we see here, this is a concentration curve. This is Bgl II plasmid representing copy numbers per cell. Our sensitivity here is less than .1.

In the positive control we get two bands. In the negative control we see no bands. You know, there is a small endogenous band here seen in everything that is just probably the mouse sequence.

But this is what I do not understand: In two of the three tumors there is a band and it is at around -- between eight and nine kb. They are slightly different in size in the two tumors. And they are not the expected 1.5 kb band. I absolutely have no explanation for what this is. If anyone could explain it to me, I would be happy to listen to any comments.

What I will say, though, is that if you look at the intensity of this band, it is around .1 genome copies per cell. If you look at this band it is well less. It is about .05 copies per cell. So what we can probably conclude from this is that if this really represents something real, it does not represent insertion mutagenesis because the copy number is so low, .1 copy per cell and .05 copies per cell. What does not make sense to me is why we do not see it in the Hind III and the Kpn and the Hind III Kpn I digest.

So, unfortunately, I do not have a conclusion slide because I do not have a final conclusion other than I think whatever is going on, it is not insertional mutagenesis.

I think you will hear data later on from Terry's lab as well as Mark's about their Southern and real time PCR data, which may bring some of this together or may make it more difficult. I do not know.

DR. JOHNSON: Okay. Thanks, Mark.

I think we have time again for one or two questions.

DR. SLY: That Bgl II cut was in what?

DR. KAY: That was in genomic liver DNA, total liver DNA.

DR. SLY: No. I mean, you were trying to cut the GUSB gene?

DR. KAY: Yes.

DR. SLY: Okay.

DR. KAY: It is a double cutter.

DR. SLY: If you had selected against retention of the GUSB that might explain.

DR. KAY: Again? I am sorry.

DR. SLY: If there were some selection against retention of the GUSB that might explain your result, is that right?

DR. KAY: Well, I do not think you can explain the result because you would expect to see -- first of all, you expect a 1.5 kb band. So even if you assume that there was a very unusual rearrangement of that that knocked out the one end of the Bgl II site and were giving you unique fragments, you would definitely see it in the Hind III Kpn I digest, which you do not.

And many of these blots have been repeated more than once. We had a limited amount of tumor DNA so we could not do as much as we wanted to and

some of the samples -- some of these -- most of these blots have been repeated twice and they have been very concordant.

DR. JOHNSON: Terry?

DR. FLOTTE: I think you anticipated my question, which was that -- do you not -- is there evidence that this is not some form of concatamer, episomal concatamer that is -- that has either been rearranged or not been faithful in terms of the ends being perfectly conserved because one of your Bgl II sites is sort of close to the end of the genome?

DR. KAY: Right. I mean, I still think it would be an unusual rearrangement but not totally unbelievable if we would have seen it in the other digest, and that is what is really puzzling to me. And, again, we repeated all these more than once.

DR. FLOTTE: Yes. It would be interesting to see all the sites laid out exactly where they are in there because I think you could theoretically come up with a rearrangement that would exclude those single cuts as well as the left hand.

DR. KAY: With no cutter you should see it or with a one cutter. Even if that one cutter is gone, you should still see a band and we do not see it and the sensitivities are the same.

DR. FLOTTE: But with the no cutter --

DR. KAY: And even --

DR. FLOTTE: I am sorry. With the no cutter you would see it if that rearranged form were integrated but if that rearranged form actually was somehow spread out in a high molecular weight concatamer it might be --

DR. KAY: Right.

DR. FLOTTE: -- a little difficult to see that way.

DR. SAMULSKI: Mark, can you give us a little information about the mouse? Is the animal model, the gene completely knocked out?

DR. KAY: It is a one base pair of frame shift or deletion that knocks out the gene product but it does not affect any of the major restriction sites.

DR. SAMULSKI: So based on that how much homology is there between the probe and the gene? What is put in the vector and the endogenous gene?

DR. KAY: We -- now you guys can answer that better than me but I can say under our Southern conditions we always used several lanes of naive mouse. We used a mouse cell line that also had the gene and if you look carefully at over exposed blots you do see a little bit of endogenous band. In some of the blots I tried to depict that by an arrow but these other bands are clearly not.

DR. SAMULSKI: Mark, can you --

DR. SANDS: Yes. I mean, there is homology between the mouse gene and human but, as Mark pointed out, it is not the best probe to use. The human probe is not the best if you are going to look for the mouse gene. Again we have done Southern blots and we have seen the same thing. It does not cross hybridize that strongly. I doubt what Mark is seeing is from the mouse gene. In fact, I do not know of a 9 kb Bgl fragment from the mouse gene. We have looked. We do not see it. By the restriction map there is nothing there.

DR. JOHNSON: Okay.

Mark, thank you very much.

DR. KAY: Thank you.

DR. JOHNSON: Is there one more question?

DR. McKEON: Mark, you showed us a bunch of different vectors,

probably over 100. Could you tell us how many of those have a promoter that is similar to the one that Mark Sands used and how many contained a lysosomal enzyme transgene? I assume Gordon Watson's did but I do not know about --

DR. KAY: Yes, Gordon and some of Kathy Ponder's, I believe, because she did those studies, I think, with you. The exact number I would have to go back and count. I do not remember off the top of my head.

I believe that Terry is going to show data using a similar promoter with different transgenes. The GUSB of Gordon Watson used a CMV promoter enhancer and it is different than what Mark has used.

DR. JOHNSON: Thanks.

We are going to move on to Terry Flotte from the University of Florida. Terry also has a dataset that speaks to long-term safety and I will ask Terry, if he can, to truncate his presentation slightly if possible so we can get back on time.

## LONG-TERM SAFETY DATA

TERENCE FLOTTE, M.D.
UNIVERSITY OF FLORIDA

DR. FLOTTE: Thanks, Phil.

I want to thank the organizers for the opportunity to present our data and we actually have both a significant amount of data about the preclinical safety question and about the integration and persistence question.

(Slide.)

Just to back up a step here, in assuming that there is some risk, which I think some people have assumed without data that there is a risk of vector induced tumorigenesis, we are assuming that the vector is probably going to be either capable of insertionally activating oncogenes or disrupting tumor suppressor genes. If this were totally a transgene effect then that would be interesting but that would not be a general consideration for changing safety monitoring in the field.

This assumption, of course, assumes that the vector does integrate within a gene in order to have a biological effect, integration outside of a gene, much less like to do that, which of course itself assumes that the vector integrates.

And so I thought we should in the course of doing -- presenting our animal data, present some data that addresses those questions. Are we observing those things happening?

(Slide.)

And I think relevant to this consideration, as Mark, Phil and others have suggested, is data on the number of integration events, which sort of starts out with a mental calculation of the number of copies per cell and then looking at the proportion that are integrated versus episomal. Or another way to look at it is the proportion of vector ends that are associated with junctions into the host cell chromosome, which, as he nicely laid the groundwork, is mapped best by Southern blotting.

But again theoretically for those thinking risk assessment, obviously 100 copies, even if the vector is integrated, 100 copies integrated as one long multimer will present statistically less of a risk than 100 independent events presumably.

Now I am going to present some data on various elements of this related to lung delivery of the recombinant AAV CFTR vector, which is the vector, as Phil pointed out, that has been -- had the most clinical use, about 70 human subjects treated with this. I will present some of the data that we have presented to the RAC in 1994 for approval of the first clinical trial.

I will present some muscle data, which is actually very interesting and talks about some host cell effects, and then we will present some liver data. I am not going to talk about the quantification of copy number that we did in Mark Sands' tumors. We will leave that to him but just to say I was amazed to see that even though we did this in an entirely blinded fashion that our copy number estimates agree completely with those of Mark Kay so precisely.

I also want to point out we did, which I am sure Mark will, that we have done quantification of other tumors that have absolutely no detectable vector DNA. An important point that some of these tumors have no detectable copy numbers at all.

(Slide.)

But let's go into our data. I am going to very -- in an almost unfairly -- capsulize here this data that we generated about the fate of vector from human bronchial epithelial cells and then from Rhesus and from rabbit studies.

We actually did studies in probably about 80 rabbits, including all the biodistribution studies in the short-term animals and so forth, but 17 animals that were followed fairly long-term, at least three months, out to two years and that were examined systematically by a pathologist.

Rhesus, we think, is kind of interesting. It is a shame they are so expensive and hard to model because I think it is important to point out that Rhesus are an experimental infection model of AAV. Phil did not mention this but we published a couple of papers on this that, in fact, in Rhesus monkeys AAV II can, in fact, persist long-term wild type virus. It can integrate with some frequency particularly persistent lymphocytes, which is actually concordant with one human report. It can be rescued. It can either replicate with coadministration of an adenovirus, a host range adenovirus that replicates in monkeys or can be rescued.

So Rhesus has some biological merit to it. Again it is -- why does it do all that? It is a natural host. It has an AAV S1 homolog that Jude's group has described.

And then we have data in human cells that admittedly is ex vivo but, in fact, it has -- we had good estimation of copy numbers by Southern blots and FISH, integration frequencies comparing FISH metaphases with FISH interphase signals, and comparing fraction in the DNA Southern blots. And essentially to summarize it, in the ex vivo studies we saw a somewhat higher integration frequency, although still less integration than total copies certainly.

In the in vivo studies which were done by putting the vector into the lung of the animals and then subsequently at the timed intervals harvesting those cells and either directly doing Southern blotting or propagating them so that we could do FISH with the metaphase preps, we are able to see a high copy number right at the site of administration but no detectable integration. Again suggesting that there is -- that the vector in the lung is mostly episomal.

It does not rule out some proportion but most -- it is certainly far different from the mechanism of a retrovirus where one active copy is one integration. Here the data is not detectable, which probably means less than 10 percent.

And then, of course, to point out no tumors were identified in any of these animals and it was a fairly large dataset there.

(Slide.)

Just to show you a little bit about what this FISH data sort of means. Here is a control here. We have wild type AAV and bronchial epithelial cells. This

was Sandra Afione in my lab and Bill Kearns generating this data. You can see the sister chromatid integration on the labeled chromosome 19 here.

But the cells from the monkeys, what you see is you see signals in interphase nuclei but you do not see signals in metaphase spreads. Again there could be differences in sensitivity here but this is very consistent with the Southern blot data showing low molecular weight episomal forms and higher molecular weight forms that appear to be concatamers.

(Slide.)

Okay. Now I want to talk about some of our IM experience. I do need to acknowledge the NHLBI, which I should have acknowledged, for funding the lung studies on a different grant. But anyway we have a lot of experience with an alpha 1 antitrypsin vector, which -- some of which has got a different promoter than the Sands' vector, and much of which has the same promoter. High doses in these studies, up to $10^{13}$ DNA resistant genomes per animal, which is about $4X10^{14}$ genomes per kilo.

Twelve animals in the original study, 36 animals subsequently.

But I want to tell you a little story here that I think is rather interesting about how these -- we think these concatamers are forming that really was a serendipitous observation in our comparison Black-6 versus Black-6 SCID mice.

(Slide.)

Now this is the expression data and, as Mark pointed out, you give a single injection. This is actually IM data. Single injection, slow up slope of expression, reaches a plateau, which goes out forever.

Now when we originally did these studies we did them both in Black-6 mice and in Black-6 SCID mice because in some of our earlier studies we found antibodies were developing to human AAT in the mice. So we did this because of concerns about immune response, which turned out not to be an issue at all.

The story I will get back to in a minute is that these mice actually genetically differ only at one locus which is the catalytic subunit of the DNA dependent protein kinase, which is involved in VDJ recombination to generate immunoglobulin and T cell receptors.

So when we get to the story about how the genome looks here it is going to be interesting.

(Slide.)

Just as Mark has done, we have looked at a Southern blot analysis and I have simplified this because we do zero cutter or one cutter, two cutter. The other forms are easier to understand but we can easily, with a one cutter that is asymmetric here, distinguish free ends of the vector.

If we consider just the tail end now, we can see free ends of the vector that are not involved in junctions to anything. Concatameric ends that are tail to tail so that you get twice the free end band size or these head to tail concatamers that were talked about before that nicely drop out of a unit size band. A one cutter will give -- in a vector genome junction will give a faint smear or a new band if it is a clonal integration.

(Slide.)

I presented this -- I mentioned this before but this data is all in these SCID mice. Two points I want to make. I will not go through all this but we can see all the different versions just as Mark has said, tail to tail, head to tail, free ends and then what appears to be integrated copies. This down shift here where you go from uncut DNA to cutting with a zero cutter that cuts outside the vector and some other

information I will give you in a moment.

Interestingly, at 18 weeks we see a lot of this free end material here, linear episomal monomers or at least, you know, free ends, which are high -- you know, account for a high proportion of the total genomes, which are shown here in the two cutter.

By 52 weeks this drops down quite a lot and it is not really accounted for by other forms and so when we have analyzed this by densitometry, essentially what we are seeing is lots of free ends early on, about 20 percent. A lot of these other forms as well, as Mark said, and -- but what appears to be a fairly high number of vector to genome junctions or junctions that cannot be accounted for anywhere else that increases over time.

What I did not focus on initially was that this is all SCID mice data.

(Slide.)

Well, we have gone back, much to our surprise, and looked in the Black-6 mice. Now you will have to look at this. There are different copy numbers in these two samples, which is because it is something to do with the fact that when we sample the muscle that is injected, we inject a large area of the muscle to optimize expression, and we do a lot of different studies.

But I will just focus you in here on the free ends. Free ends are very abundant in the SCID mice, not detectable in the Black-6. You might say, well, that is really a big difference here but you can look at the head end again. In this -- if you use a CMV probe out here, very clear free end band here, no free ends here, and again here in this case this two cutter actually -- the probe straddles this so lots of free ends here. No free ends in the normal mice.

(Slide.)

What could this mean? Well, you know, I -- it is not as simple as this model. This is not an all or none phenomenon and I put this up here as the beginnings of a model that goes to a step one beyond where Mark has nicely outlined the issues of how you get to a double stranded form and beyond -- and the fact that there is concatamerization but how might this concatamerization occur and why would the presence or absence of the catalytic subunit of PK have an effect here?

Well, one, you know, fantasy here might be that in the presence of PK it might actually interact somehow specifically with the ITRs or perhaps just with free ends of DNA. We have not formally proven this is an ITR interaction. And that would favor, in fact, a fairly rapid evolution from these forms to these concatamerized forms, which would form regardless of the orientation so you would not really care here whether it was head to tail, tail to tail or so forth.

But in the absence of PK it is clear we are observing a lot of linear monomers very far out, which is very different from what we see in the Black-6 mice. And then the -- and then the progressive accumulation of these integrated forms.

(Slide.)

Now I have put this up here as a -- just to get to a basic question because somebody, who is a smart person, asked me, "Well, why would you think that AAV would not form tumors?"

Well, one reason might be if this DNA is interacting with a component of the host cell nucleus and PK is really ubiquitous in various cells. And using, you know, this mechanism, which it actually is a -- it does have DNA ligase activity -- to form a relatively safe, to be provocative, a relatively safe episomal or concatameric

form.

Now again you could still get some integration on these ends here but, you know, your risk per delivered genome would be very different.

(Slide.)

Now I am going to present some very preliminary data that might support this model and I cannot go through all this in detail here but this is an in vitro integration assay and I just want to point out here that if you focus in here, we have done this with and without rep for other questions we have about how this might function in terms of wild type, but in terms of recombinant you focus right here on minus rep lanes.

If you in vitro add in PK you inhibit the integration, the readout here for integration is this unique band, you inhibit the integration, you decrease the PK some, you get more integration, you go to just the nuclear extract alone and you have this amount of integration. Then you remove PK with an antibody and you further enhance integration.

(Slide.)

Now could this be some kind of artifact? Well, we have also looked at some stable cell lines that are available. The MO59J cells, which are PK- or the K cells that are PK+, again confirm that by Western blot, J cells totally PK-. K cells actually over expressing PK as compared to HeLa cell extracts.

And again it is pretty simple here. K cells have DNA PK, much less integration. J cells lack PK, much more integration. And the same trend with or without rep but it is really a different story.

(Slide.)

So to come back to this again. You know, perhaps integration can be strain specific for a number of different reasons. And essentially to say the host -- host strain plays a big role in this risk assessment. Now, you know, I do not have any reason to say that the MPS VII mice have an absence of PK but the point is just simply that the host may have a mechanism to deal with these genomes, which results in the observed -- what might be considered unexpectedly low incidence of mutagenesis.

These have never been successfully used to do mutagenesis studies. I think it is worth pointing out historically that a lot of people have thought, well, if this is an integrating vector, and without rep it integrates randomly, wouldn't this be a great way to generate insertional mutagenesis libraries. And everybody has failed to do that so far. I think that is an important point, you know, that insertional mutagenesis is just not seen.

(Slide.)

Now the next part, I want to talk about the liver. We have talked about lung. We have talked about muscle. I want to talk about the liver now. We again have to acknowledge the NIDDK, who has funded several investigators here in a P01 -- it is not here, down in beautiful sunny Gainesville -- to look at AAV delivery to the liver, including many people who contribute or just donated their data to us to look at in this safety comparison. Phil Lapis, Ken Berns, Barry Byrne, ourselves, and others.

Like Mark Kay's data, we see this slow rise. We see a peak between two and four weeks that is sustained for a long time. Levels of expression are very high. We have done experiments with -- all this data is with the CVA promoter, the CMV enhancer, chicken beta actin promoter with this part of the rabbit beta globin entron. The same cassette. We actually got the cassette from Mark Sands. And we have done doses that are shown on here in infectious units. $3x10^{10}$ infectious units,

which is about $10^{12}$ genomes, which in the mouse is $5X10^{13}$ per kilo.

(Slide.)

I have probably already gone over my time so I will not go through our Southern blots in great detail again, except to say that what we find mostly in these are, in fact, by virtue of this one cutter in these now intact Black-6 mice are evidence of these concatameric vector to vector junctions, no evidence of free ends, and not -- no genomes that we have to account for by integration by this general screening sort of method. We do not have FISH data yet from the liver but we are getting there.

(Slide.)

So again we would say that probably less than 10 percent of genomes are integrated. Very consistent with Mark's data which is reassuring but we are happy about it.

(Slide.)

Now I have another table like this from our liver experience. I did not do this with our lung and muscle but we were requested to do this and, of course, you cannot read it. But the only point is that we have a bunch of studies with newborn IV animals, a bunch of studies with adult, whatever you call adult. Actually weanling, three to six week young mice by portal vein injection. And then a bunch with IV injection into adults. Most of the vector there also goes to the liver.

We have done a number of different strains. We have not done MPS VII animals. We have done a number of different vector cassettes. Some different promoters but CB keeps coming up over and over. We use it more because it works better. This was not intended as a safety study of the CBA promoter. Just we wanted to get our genes to work.

The doses have been quite high and you cannot read these but our newborn doses are typically in the $5x10^{9}$ infectious unit range, which is about $10^{11}$ genomes, which is in a newborn mouse that is about a factor of 500. So that is $5X10^{13}$ per kilo. And follow it out for various time points.

(Slide.)

Now let me just summarize this data. Our experience with liver delivery is 137 animals that have been systematically examined by Jim Crawford in his core lab. Of those, 43 were newborn IV injected. Many of these, 40 percent of these or more, were followed out for about a year or longer and many actually have been over 13 months.

Mark has shared his data with us. Mark Sands. And we -- Ron Marks in our group did a meta-analysis. If you look at this number versus the number of tumors he observed, that is very statistically different as a dataset. If you just look at the newborn IV injected, it is still very statistically different. And if you just look at the long-term animals the significance is still there. It is not as dramatic but it is still there.

So what we can only say from this is that with similar vector cassettes and similar higher doses we see a very different and essentially nondetectable risk of tumorigenesis.

(Slide.)

So, to conclude, in a number of sites of injection, the largest portion of genome copies are persistent forms that are not involved in junctions with the host genome and that is a carefully worded conclusion because again it could be part of a concatamer that is hooked to the host genome at the ends but most of the copies are not associated with the junction into the host genome.

The incidence of tumorigenesis with recombinant AAV is not detectable in our studies. I have not tabulated the total numbers but are really literally hundreds and hundreds of animals over the ten years we have been doing in vivo studies by endobronchial, intramuscular, intravenous, intraportal routes up to $4X10^{14}$ per kilo.

This dataset is statistically different from the Wash. U. dataset, which has been handled very carefully, and so I suspect that there is something different in the experimental design to account for this. The vectors are basically in common but there is something else about the experiment perhaps. That at least statistically seems to be the case.

And just to say that in our hands there is no objective evidence that recombinant AAV poses a risk of carcinogenesis that is detectable with the number of animals that have been done, which is a significant number.

(Slide.)

I have to acknowledge many people here. I have to acknowledge Sihong Song who did most every Southern blot you have seen here and various other collaborators who have donated their data. Ken Burns' lab with the in vitro integration studies. Nick who sort of brokered this whole thing. I have to also acknowledge Barry Carter. All of the original lung studies were done in his lab on the Bethesda campus or in collaboration with Targeted Genetics Corporation, who has been our partner all the way along. And various other collaborators at Hopkins. Bill Kearns, who did FISH for many of our studies or taught us FISH. And then the funding sources.

So I will end there. Thank you very much.

(Applause.)

DR. JOHNSON: Okay. We have time for a few questions for Terry.

Mark?

DR. KAY: Terry, I noticed that in some of your experiments you used glycogen storage. I think that was Type 1. You were treating those animals. Glycogen storage Type 1 deficient glucose-6 phosphatase.

DR. FLOTTE: Right.

DR. KAY: I mean, I want to make a point and then ask you a question. I mean, in human disease almost all those individuals develop hepatic carcinoma and the mechanism is not totally clear but it is believed to be or hypothesized to be due to chronic insulin signalling. I wondered if you or anyone else that you know about has data whether the mouse model develops hepatic tumors.

DR. FLOTTE: That is a good question. We actually excluded this -- the data in the MPS -- I am sorry. The glycogen storage disease Type 1 we ended up excluding here because those slides were not systematically examined by our core. Actually that was data that was given to me by Barry Byrne. It was actually done on the NIH campus by Janice Chiu. And, in fact, there is an incidence of hepatic -- hepatocellular carcinoma development across the board. It is well described in this model. As you have pointed out to us, it occurs in the patients.

The animals actually do not stay alive very long. The longest term data is about two or three weeks after vector injection, you know. So there is really not meaningful data with regard to any vector effects. So for that reason the data is really short-term rather than long-term. It was not processed through our core. We did not put that in here but we made the vector associated with those studies.

DR. JOHNSON: Phil?

DR. NOGUCHI: Phil Noguchi, FDA.

Just a comment and a question. Just to caution everyone that lessons we learn from one type of virus may or may not really translate to the other. I think your last statement, the only quibble we might have is saying that this is evidence that carcinogenesis related to integration of AAV vector has not been demonstrated at least in your data. There certainly may be other forms of carcinogenesis. As has been pointed out, AAV as an instigator of -- itself of carcinogenesis anywhere has just not been demonstrated. If we had a retrovirus, one obvious question is if you have a multiple hit you do get tumorigenesis. So it is just saying that that conclusion is correct if we add the qualifier of integration not being -- and tumorigenesis not being demonstrated in your data.

The second question is in the -- was in the PK- animals or the ones where you found more integration in the absence of the enzyme. Did you look at wild type AAV? I am just thinking out loud. Would you -- might you expect to get multiple integration sites say at -- instead of in one chromosome, in other chromosomes?

DR. FLOTTE: Okay. This is a good point. We may end up -- there may end up being reagents to do that experiment ultimately. Mice do not naturally have a homolog to the AAV S1 site but there are some transgenic strains that are available that are on a different background. They are not SCIDs and they are not otherwise in the Black-6 background. So there is really not right now a mouse model available to do the kind of study that you are suggesting, although there are strains that could be crossed to create the strain that you are interested in or a transgenic could be made in the SCID background.

DR. SAMULSKI: Terry, I would like to get you to help us a little bit. In all of the animals you looked at, how many animals, if any, got the same dose that Mark was using via the same route of administration at the same age?

DR. FLOTTE: Well, I do not want to go back to the slides. I believe the total is 43 animals that got newborn IV injection. All of those animals have the same vector cassette, AAV ITRs and the CBA promoter because we were given it by -- given to us by Mark. And those were -- that is the newborn IV head vein injected animals.

The doses in those instances generally are -- this particular vector, the alpha antitrypsin vector packages very efficiently and so we have got higher titers of that and the typical dose range was about $5X10^{9}$ infectious units or $10^{11}$ genomes per animal, which is about 20 to 50-fold higher than the Wash. U. data.

DR. SAMULSKI: So technically about 20 to 50-fold more virus put in the same route of administration at the same age and then what is the length of time that these animals were looked at for whether or not they had any --

DR. FLOTTE: I am going to put this back up and show you this.

DR. SAMULSKI: I think it is just important because it gives us a clear example of comparing side to side.

DR. FLOTTE: That was the meta-analysis data that we showed.

(Slide.)

Okay. So I have the lost the ability of the pointer here for some reason.

All right. But anyway the -- here we go.

The newborn IV injected total was 43, 22 that went out past 11 months or later. Actually eight months was the cutoff when you started seeing tumors but roughly half of these. And these -- actually all the ones that went past this time point

at this route of administration were out well past 13 months. Some to beyond 18 months. And both this number and these numbers are statistically different than Sands' dataset.

Actually let me correct that. This number and these numbers, zero of these numbers are statistically different. If you narrow it down just to past 11 months, zero to 22, that is just at the boundary of statistical significance. Like .06, I believe it is, or something about around there.

But, you know, as Mark Sands alluded to, long-term exposure of the liver -- I mean, if you think about it, you inject in that newborn period versus three or four -- three to six weeks later, I mean you are still having long-term exposure of the animals. But zero of 43 here, I think, as we point out, is very different from the total experience of six of 59. But if you hone it down to zero of 22 versus six of 18 in his studies that are out past these time points it is just at the boundary of statistical significance. Zero looks different than six, you know. I mean, if you take a nonstatistical approach when you are talking about 20 animals in each side of the experiment.

DR. SAMULSKI: So, Terry, as far as the difference, it was a different transgene and a different animal model?

DR. FLOTTE: Those are the only two differences that I can perceive right now, although Mark may think of something else, but different transgene and a different strain of mouse.

DR. JOHNSON: Well, thank you, Terry.

We are going to stop before the break.

I have been asked to announce that everyone needs to register if you have not done so already, please. We are going to reconvene at about ten after 10:00 with Mark Sands.

Thanks.

(Whereupon, at 9:56 a.m., a break was taken.)

DR. BREAKEFIELD: Can we start up again here?

Well, we would like to start up again. I know there is a lot of excitement and a lot of things to talk about but it would be nice to do that in the roundtable discussion so we can all hear what -- everybody's ideas.

The next talk I am sure we all want to hear. I would say just from some of the conversations I have heard, this is a brave man who speaks his conscience and conviction, and has taken a little bit of heat for it but I think that this is really the way the process should work and I congratulate him on sharing his data with us and allowing a nice open discussion format to talk about it.

So Mark Sands is going to talk about long-term data using AAV GUSB vector in MPS VII.

## LONG-TERM DATA USING AAV-GUSB VECTOR IN MPS VII

MARK SANDS, Ph.D.,
WASHINGTON UNIVERSITY SCHOOL OF MEDICINE

DR. SANDS: Thank you.

Actually I am not sure if I really want to thank the organizers for inviting me.

(Laughter.)

I seem to be the outside person.

As everybody, I am sure, is aware, the data I am going to show today

will present some of the toxicity that we have observed in our animal model following AAV mediated gene therapy but before I actually get into the discussion of the tumors we discovered, I first want to give a very brief overview of the experimental mouse model that we used and the experimental design that led up to the discovery of these tumors.

I would like to point out at this particular time that this study that we did was not designed as a toxicology study. It was really designed as an efficacy study to look at the effects of AAV on the disease. Okay.

(Slide.)

So for those of you who are unfamiliar with lysosomal storage diseases, which probably many of you are. They are fairly rare disorders.

Lysosomal storage diseases are generally caused by deficiencies in a single lysosomal enzyme. In the absence of any one of these lysosomal enzymes, the substrates that are normally degraded in the lysosome cannot be degraded and they accumulate to very high levels in the cells.

Now this accumulation of un-degraded substrates can lead to a broad spectrum of clinical symptoms. Often times including auditory defects, visual defects, severe skeletal dysplasia, visceral lesions and severe cognitive defects. And also children with these diseases, as with our mouse model, have a dramatically shortened life span as well.

(Slide.)

So over the last 10 or 12 years to try to better understand these diseases and to try to develop novel therapies for this group of diseases, we have concentrated most of our efforts on a murine model of one of these diseases called mucopolysaccharidosis Type VII. As has already been pointed out today, MPS VII is called by deficiency of beta glucuronidase. In the absence of this activity the substrates that accumulate are complex glycosaminoglycans. For example, chondroitin sulfate, heparin sulfate and dermatin sulfate.

In the absence of this enzyme or in the -- with the accumulation of these un-degraded or partially degraded glycosaminoglycans you can get a wide spectrum of clinical features. This side of the slide shows our murine model of MPS VII.

I have got listed on here the clinical symptoms associated with this disease, which again include severe behavioral abnormalities, corneal clouding, retinal degeneration, profound hearing deficits, cardiac valve defects, intralysosomal storage throughout most cells of this mouse, severe skeletal dysplasia and dramatically shortened life span. Virtually all of these clinical symptoms are shared with the human disease, MPS VII.

(Slide.)

So again we have used this model to try to better understand the pathophysiology of the disease and again to develop novel therapies. Over the years we have experimented with a number of therapies, including bone marrow transplantation, direct enzyme replacement, a number of different approaches, and most recently we have been using a gene therapy approach utilizing adenoassociated virus vectors as the gene delivery vehicle.

(Slide.)

Without going into all of the preliminary data that we generated, we first constructed an expression cassette, which is schematically represented here, in the hopes that we could accomplish two things. First of all that we would get relatively

high level beta glucuronidase expression and we chose the expression cassette that we did to not only get high level of expression but also to try to get ubiquitous expression. So the expression cassette we used has already been at least superficially described this morning.

It is composed of the cytomegalovirus enhancer, chicken beta actin promoter, the first entron from chicken beta actin, the human beta glucuronidase cDNA, and then that is followed by the rabbit beta globin polyadenylation signal.

This is an extremely powerful cassette, expression cassette, and it functions quite well in almost every cell type that we have put it in so far.

So we created this adeno-associated virus vector, tested it in cultured cells. It seemed to work fine. And did a couple of preliminary experiments in vivo to show that it worked.

(Slide.)

And then we did a very simple minded experiment and just took newborn MPS VII animals and these were animals that we identified at birth and injected either on the first or second day of life, and we gave them an intravenous injection of virus.

In our first study, which is summarized here in this panel, what we showed is that we can get beta glucuronidase expression in a wide variety of tissues. We have a very sensitive histochemical stain for beta glucuronidase activity and anything that is red is where beta glucuronidase activity is localized. And you can see there are lots of positive cells in heart, neurons of the brain, meninges, retina, liver, and in fact I could add a number of other tissues in here. We saw beta glucuronidase expression in many cell types. This distribution and level of activity was sufficient to virtually eliminate lysosomal storage in many tissues in these animals.

So this was our initial finding following intravenous injection of virus at birth but this study was limited in that we only looked at the biochemical and histopathologic consequences of AAV mediated gene therapy and the study was limited to only about four months. So we set up a much larger study and designed it to ask the questions, do we get clinical improvements along with the histopathologic improvements that you can see here and what are the long-term effects of AAV mediated gene therapy.

(Slide.)

So we identified 59 animals, newborn MPS VII animals, and injected them with the same thing. On the first or second day of life with a dose of approximately $1X10^{8}$ infectious units per mouse. A newborn mouse weighs approximately a gram. And then put those animals on the shelf and certain animals we sacrificed at different times to look at the biochemical and histopathologic consequences of this therapy and let some animals go long-term. Other animals we also measured clinical parameters.

(Slide.)

And this slide really nice represents the clinical response that we saw. This is a normal animal here. This is an uninjected MPS VII animal and this is an MPS VII animal that received a single injection of virus the day it was born. Clearly the phenotype is dramatically improved. But when we also measured functional parameters such as electroretinograms, auditory evoked brain stem responses, body weight, all these parameters that we can measure, these animals are significantly improved in all of those categories. So from an efficacy point of view, the AAV mediated gene therapy worked very, very well with respect to the disease, the MPS

VII.

(Slide.)

So as the study progressed, we looked at the expression level of beta glucuronidase and very much like what everyone else has seen with AAV, after an initial period where there was either increasing expression or some variability in expression, the level of expression is very consistent all the way out to about a year-and-a-half in multiple tissues in these animals.

So what happened is out here at approximately one year of age we sacrificed three animals for biochemical and histopathologic characterization and discovered that at one year, at least in the animals we sacrificed, there were no gross lesions in the liver or any place that we looked. So we compiled all this data and sent it in for publication, and during the process of review and acceptance, a year-and-a-half time point came up. We sacrificed additional animals and that is when we begin to see the toxicity that we have observed.

(Slide.)

So at a year-and-a-half we sacrificed -- we had five animals remaining. Five newborn MPS VII animals that were treated with AAV at birth. Five animals were still alive. We sacrificed three at a year-and-a-half and immediately as soon as we opened them up we noticed that there were relatively large tumors on their livers ranging from between one to two centimeters in diameter. So we immediately stopped the study, went out to the mouse colony, got the remaining two treated animals, looked at them, and one out of two of those animals also had lesions on their liver.

(Slide.)

So at this point, at a year-and-a-half, we had three out of five animals that had hepatic lesions. So what we did then is we retrospectively went back and looked at all the animals that we had salvaged during the longevity study. These are animals that had died spontaneously and we managed to save the carcass before it had time to decompose too badly.

When we compile all this data, we see that we also found additional tumors. Interestingly, the very first tumor we discovered was in an animal that we sacrificed at about eight to nine months of age. Now at this point we had already sacrificed over 40 animals and had not seen any evidence of toxicity. So finding one animal at 35 weeks did not upset us too badly at that point but we kept it in the back of our minds.

If you remember, at one year of age, we purposely sacrificed a number of other animals and saw no gross lesions. However, on a retrospective examination of some of the animals that died spontaneously, two animals died at approximately one year of age spontaneously as part of the longevity study. Those animals also had tumors. One had a hepatocellular, a low-grade hepatocellular carcinoma. The other animal had an angiosarcoma.

So now in the entire study out to a year-and-a-half we have at least six animals that had some sort of cancerous lesion. Again the primary lesion is hepatocellular carcinoma. However, we also did see angiosarcomas in two cases.

(Slide.)

So to try to better understand this -- well, before I actually go into what we have done since then, I would like to show some of the histopathology of these tumors and I will freely admit right now I am not a histopathologist and if I get some of the jargon incorrect, I apologize.

But this is a section -- I know this is probably difficult to see in the

back but this is a section of one of the tumors from one of the hepatocellular carcinomas. Basically what you can see if you were a trained pathologist, you can see that the hepatocytes are very dysplastic. Meaning that they vary greatly in size. Many of them have multiple nuclei. And we have a very high fraction of cells that are undergoing mitosis. In fact, in this particular slide we see two cells right there that are in the process of undergoing mitosis. And in every single animal that we examined that had a liver tumor, the histopathologic findings were identical in all five animals.

(Slide.)

Now with respect to the angiosarcomas, this is a section of spleen from one of the animals that had an angiosarcoma. The primary lesion was in the uterus but this is a metastases that is in the spleen. And the salient features here are that the tumor is here and basically what you see are these pools or pockets of erythrocytes, which are characteristic of angiosarcomas. And on higher magnification examination of these tumors, they look very characteristically like angiosarcomas. And remember two animals had angiosarcomas.

(Slide.)

So in an attempt to try to understand what the mechanism is, if you just look at this data and think about it for a while, you can generate a large number of hypotheses that could explain the formation of tumors in these animals. One of which is simply that the MPS VII animals are predisposed to carcinogenesis or tumorigenesis. Unfortunately, these animals do not live long enough. At least fifty percent of the animals are dead by six months and no untreated animal has ever lived beyond a year.

And given the timing that we saw these tumors, these animals -- these untreated animals really do not live long enough to see these tumors and, in fact, over all the years that we have looked at these animals we have never discovered a hepatic tumor in one of the untreated mice.

(Slide.)

So what we have done is we have looked at a number of different animals that either we had or that were provided to us for examination. And what I will tell you right up front is although we are looking at a number of animals here, there are not merely enough animals in any one of these groups to really make any definitive statements about the mechanism of tumorigenesis or the lack of tumorigenesis in any of these animals but this is what we had on hand and I will present it as such.

These are the animals which I have already presented. Animals that had -- from AAV treated animals that had hepatocellular carcinomas or angiosarcomas.

This group right here, these two groups actually, represent MPS VII animals that received a bone marrow transplant on the day they were born. These animals were provided to us by Jane Barker up at the Jackson Lab. Unfortunately, she is snowed in up in Bar Harbor and she could not be here today but she graciously provided those animals to us for analyses.

There are 24 animals in this cohort. All of these animals went beyond one year and six out of the 24 simply got bone marrow cells the day they were born with no radiation, no ablation. Eighteen of those animals received 100 rads or sublethal radiation the day they were born followed by the same injection of bone marrow cells. And in all of those animals the interesting finding was that a high

percentage of them developed cystic ovaries, which it is not exactly clear why that is at this point, and actually this -- we also saw one animal with a lung tumor. This number two here. One of the tumors was accidentally identified as a tumor on gross necropsy. It turned out to be a pneumonia.

So of these 24 animals well beyond a year that received bone marrow transplantation at birth, only one animal had a tumor and that was a lung tumor. No hepatocellular carcinomas or angiosarcomas for that matter.

Now we also a few years ago generated a transgenic animal and this transgenic animal actually serves as a pretty good control because we purposely made this animal that harbors the exact same expression cassette as our AAV vector. The only difference is it does not have the ITRs on it. So it is the CMV enhance, beta actin promotor, human beta glucuronidase. And we also made this transgenic on an inbred background so instead of doing the typical F1 hybrids, we did it on this same inbred strain so that we could do transplantations without interfering with any immunologic barriers. So this actually serves as a pretty good control just for over expression of beta glucuronidase.

Well, we did not have a lot of these animals on the shelf because we did not -- this was an unexpected finding but we did have six animals out in our mouse room that ranged in age from 48 to 60 weeks of age. We sacrificed those animals and we found no gross lesions in those animals anywhere. No hepatocellular carcinomas. No angiosarcomas.

And, also, as part of the longevity study, from the AAV gene therapy experiment we had eight uninjected normal siblings that were carried along for the longevity study just to fill out that curve. We sacrificed those at about a year-and-a-half. None of those animals had any obvious lesions either.

So these data would suggest, again very small numbers but at least it would suggest that there is not a really high incidence of tumors in any of these treated animals with the exception of the AAV treated animals.

(Slide.)

Now as Terry mentioned, Terry had developed a real time PCR assay for our particular expression cassette. Not beta glucuronidase but the -- I believe it is a CMV enhancer beta actin promoter region, which is obviously unique to that expression cassette.

So in a blinded fashion we sent him DNA samples either from the tumors from these animals or from normal looking liver tissue from the same animal. Okay. So these are animals that had tumors. Normal looking liver tissue and portions of the tumor. And, as Terry mentioned, we basically get a mixed bag of results here.

These last two animals -- these two animals represent MPS VII animals that were injected with AAV at birth but had no tumors at a year-and-a-half and we just examined their livers for the presence of the AAV genome. Interestingly, we saw genome there. Well, actually not unexpectedly. We expected to see the genome there. But, interestingly, these other animals had the tumors. This is the animal from 35 weeks and this is a little confusing because there is apparently no AAV genome or no detectable genome in either the tumor or the normal looking liver, although we still saw expression in this animal so this is somewhat confusing.

However, these three animals, you can see that in at least two of them we saw evidence of the AAV encoded genome in the tumor and in the normal portion of the liver. Now the amount of genome in the tumor exceeds that -- exceeds what you see in the normal portion. However, you see this other animal here which also

had a tumor. There is no evidence of AAV genome in that tumor but there is genome in the normal section of liver.

Interestingly, these two animals where you get a value of .1 AAV copies per cell and approximately .08 copies per cell, these are the two animals that Mark Kay presented this morning and this is consistent with his data showing that this animal had about .1 copies and this animal had about .05 copies by his Southern analysis. So there is certainly concordance here.

But again you see some tumors that have no evidence of AAV gene in them so I think, although not completely definitive, what this at least would suggest is that the tumorigenesis we have seen in these animals cannot be explained by a very simple model whereby you get an integration of that and then a clonal expansion of that transformed cell because in that particular case what you would predict is that your numbers here would be one or greater. This does not formally exclude AAV as a causative agent but again at least for a very simple model of integration and then transformation these data, as well as Mark's data, are not consistent with that.

(Slide.)

So it was suggested to us that perhaps the hepatic tumors were due to an increased hepatocyte replication due to the disease. So to try to address that issue we examined at least three animals from three different groups. One group would be untreated MPS VII animals, another group was untreated normal animals of the same strain, and the third group were animals from our transgenic colony. Again the same strain of mouse and the same expression cassette that is in our AAV cassette. So we examined three animals from each of those groups.

And the first thing we discovered is that when you look at the livers by H&E staining you see these occasional aggregates of what appear to be lymphocytes, at least by H&E staining. This is a consistent finding in all three groups. All three mice in all three groups have about the same frequency of these aggregates of cells that look like lymphocytes.

The only difference we discovered was that -- well, also before I get into the difference, also when we counted the number of replicating hepatocytes there was no difference between the normals, mutants or transgenic animals with respect to replicating hepatocytes. The only difference we discovered was that in the untreated mutant animals these aggregates of cells that appear to be lymphocytes you would find an occasional aggregate where you see BRDU staining, suggesting that there is DNA synthesis and replication going on.

We do not understand the significance of this finding and there is not a lot of these in the liver but it is a difference between those three groups. And, again, we do not understand the significance of it right now but so far that is the only difference we have been able to uncover.

(Slide.)

Now, finally, what I would like to do is put this study in perspective and put it in the context of the other studies that we have performed, we and other groups have performed over the last 12 years. And this is a compilation of some of those studies, starting all the way back from 1992 up to some rather recent studies, involving different forms of therapy in MPS VII mice.

And what you can see is that there is a relatively large number of animals that we have examined over the years with various types of therapy, either bone marrow transplantation initiated in adults or bone marrow transplantation initiated in newborns, or direct enzyme replacement in newborns or a combination of

enzyme replacement and bone marrow transplantation.

There is a fairly large number of animals here and even animals that go beyond 32 weeks of age, there is at least -- what is that -- 27 animals that are relatively long-term. And in all of those cases, with one notable exception, there were no gross lesions observed in any of those animals.

This one exception was in an animal that received 600 rads of radiation. It was a young adult animal who received 600 rads of radiation and bone marrow transplantation. And at about 298 days of age -- I think that is right -- or 28 weeks -- this animal developed a subcutaneous keratoacanthoma. Okay. But still in none of these animals have we ever observed any hepatocellular carcinomas or angiosarcomas.

So the data are still confusing to us and we have still no evidence that would suggest a mechanism for the tumorigenesis here. We have no evidence that would suggest that AAV is the causative agent. We have no evidence yet that the disease -- that these animals are predisposed to tumors either so we are still confused.

And I think with that I will end and take any questions.

(Applause.)

DR. BREAKEFIELD: Could we have the lights back on?

DR. CRAWFORD: The fact that much of your work has been done with bone marrow transplantation models raises an intriguing possibility and an aspect of research that is actually moving forward very rapidly, and that is that the bone marrow cells are actually repopulating the liver in that model.

And in work that Marcus Grompy has performed with the FAA mouse he has been able to show over 70 percent reconstitution of the liver. And again this is as much of a question as anything else, to the extent that the virus may transduce five percent of the cells, whereas a bone marrow transplant model might reconstitute most of the liver over a 12 to 18 month period, one can argue or at least posit that residual hepatocytes which might be clonagenic are more abundant in the vector model than they are in the bone marrow transplant model.

It is, in essence, bringing together the stem cell transfer to the liver field of research and the vector field of research and raising the posit that residual hepatocytes may be more abundant in the vector model.

DR. SANDS: I am not sure I understand.

DR. CRAWFORD: Well, the --

DR. SANDS: What I can tell you is that in the bone marrow transplantation experiments that we have performed -- I am familiar with all the data on the plasticity of hematopoietic stem cells and all this sort of stuff -- we have never really seen much evidence that following a bone marrow transplantation either in adults or in newborns that we actually get a significant number of donor-derived hepatocytes in those animals.

DR. CRAWFORD: Okay. That is the question.

DR. SANDS: We see almost complete replacement of the kupffer cells but we have never seen any real evidence of hepatocyte reconstitution. I am not sure that gets at your --

DR. CRAWFORD: Well, it gets to the question of whether you have, if you will, deficient hepatocytes which remain, which may, in fact, be the source of your tumors.

DR. SANDS: Right. So I honestly believe in our bone marrow transplantation experiments the hepatocytes that are in those animals, vector bone

marrow transplant, are MPS VII derived hepatocytes.

Now they do not have a lot of enzyme activity. With our histochemical stain you can section the livers, there is very little enzyme activity in those cells, which is different from our AAV experiments. The hepatocytes have an enormous amount of enzyme activity in them. But, also, in the bone marrow transplantation experiments there is also no evidence of lysosomal storage in the hepatocytes either so the amount of expression we get in the kupffer cells that are repopulated in the liver is more than sufficient to eliminate any lysosomal storage in the hepatocytes.

So I think they are still from MPS VII mice but they do not have the hallmark of the disease. I am not saying they are perfectly healthy but they do not have the -- you know, the microscopic lysosomal distension that is characteristic of this disease.

DR. MUZYCZKA: Mark, you mentioned the real time PCR data. Did you also do some Southerns?

DR. SANDS: Yes.

DR. MUZYCZKA: And a corollary to that is did you actually ask whether the tumor, which presumably is clonal, whether they were -- those hepatocytes were expressing the gene?

DR. SANDS: To answer your first question, we also did Southern blots very much like what Mark Kay did and we used a single cutter and a no cutter, and our Southern blots are not quite as sensitive as Mark's but in all the tumors that we looked at, very consistent with Mark's data, we had one cutter and no cutter, we saw no evidence of integration of AAV.

And what was your second question? I am sorry.

DR. MUZYCZKA: Was beta gluc expressed?

DR. SANDS: Yes. We actually have not done RT/PCR on the tumors. The only evidence I have to address that question is we took one of the tumors -- and, again, you have to understand this took us by surprise so we really were not prepared to analyze these in the best way possible.

We took one of those tumors, embedded it and sectioned it, and did our histochemical stain on that sectioned tumor. And what we saw was that the smaller cells, which look like maybe kupffer cells or some sort of endothelial type cell, had a lot of enzyme activity in them, which could be simply from uptake from the serum. There is an enormous amount of activity in the serum and that activity is taken up preferentially by the kupffer cells or endothelial cells.

We also saw enzyme activity in the hepatocytes but not at the same level that I would expect if it were actually transduced and expressing the vector. So I cannot give you a definitive answer but it was not this tumor that was just basically pumping out a bucket load of beta glucuronidase. It was not that case at all.

DR. BREAKEFIELD: I think we will go back and forth between the table and the audience, and if you could just identify yourself?

DR. _____: I think the characterization of the tumors is quite well done but how well characterized is the vector prep? It is a trivial question but how do you know --

DR. SANDS: It is not a trivial question. Actually I feel very fortunate in this particular case. This was such a large study that my relatively small lab -- it would have taken us months to make enough virus to do this study so we actually contracted out to Nick to make these viral preps and these preps were made by cotransfection of the transfer vector and PDG, which I am sure you are familiar with

PDG from Miriam Kleinschmidt's work. It was done by a cotransfection method. It was also purified by aldixonal gradient centrifugation and heparin agarose chromatography.

And then it was characterized at least enough to say that there was very little, almost undetectable levels of wild type AAV, and there has never been any adenovirus detected with this production system. So it was good virus. In fact, in my opinion it was much better virus than what we used for our initial studies.

DR. _____: Yes. But that is only looking at certain biological criteria and the protein profiles. There are other things that obviously are biological effects which would --

DR. SANDS: Sure.

DR. _____: -- not necessarily come up with these assays and also the other animal data that were presented earlier were presumably from different -- perhaps used different protocols for the processing.

DR. SANDS: Well, I believe, and Terry can correct me if I am wrong, I think Terry's virus with all the animal data that he showed --

DR. _____: Right.

DR. SANDS: -- the viruses were made in exactly the same way, I think.

DR. FLOTTE: Yes, that is true of all the liver -- the liver -- you know, the lung data was older with other methods but the liver data.

DR. SANDS: So in that respect our data is comparable to what Terry presented at least in the way the preps were produced.

DR. BREAKEFIELD: I think later in the panel discussion the issues of vector production are very important to evaluate across, and we are going to go back and forth so, I guess --

DR. KAY: I had a question about the issue that I was surprised by the real time PCR that there was so little vector in the liver and the question is whether you could be getting gene expression outside. I mean, Karen Gessler's lab shows in neonatal mice when you inject AAV that you get the peritoneum lighting up.

But as a corollary to that, I guess, my question is if you ever assumed that there is a very small percentage of hepatocytes that are transduced, taking the AAV transduced livers, measure total enzyme activity in the liver, compare it to that of a transgenic, and work back and say how much enzyme per cell is being made. I know it is not a perfect assay to see whether it is in the same ball park as --

DR. SANDS: No, we have actually never quantified that in that way but that would be -- that would be a good approach because our histochemical stains are not quantitated. When you see a red cell it is not quantitated so I do not know the answer.

DR. KAY: And one other just corollary in regard to the Southern and the PCR data. Do you know what -- roughly what proportion of the cells in the HCC appear to be hepatocyte derived because it is an important issue for clonal integration events if they are diluted out with lots of other cell types that --

DR. SANDS: The tumors appear to be primarily hepatocytes. Now when you look through you can see occasional lymphocytes and you certainly see things that look like kupffer cells or endothelial cells but they are primarily -- the vast majority of cells are hepatocytes. Dysmorphic hepatocytes but clearly hepatocytes.

DR. BREAKEFIELD: I guess we will go to the audience. Louise?

DR. MARKERT: Louise Markert, Duke University and a RAC

member.

One hypothesis you have put forward is that there is an increased incidence of tumors in the deficient mice and so much has happened in immunology just in the last few years in terms of ability to assess the immunology in so much more detail compared to previous years, what with immunoscopes, flow based cytokine analysis, TRECs, looking at naive cells more, aside from the NK function and T cell function antibodies, and responses to neo antigens.

What is known in the human situation or has there been a relook at that population when they are born to see can -- with all the new techniques available are there some immune deficiencies in that animal or in the human that, although may be subtle, may lead to an increased incidence of tumors. If the patients were to live long enough certainly there are millions of immune deficiencies where tumors are very prevalent.

DR. SANDS: Right. That is actually a very interesting question and it was last year we actually published a paper where we described an immunologic defect in the MPS VII mouse and when we went back and did a literature search trying to look at the human data and what was known from clinical studies, there is really no description in the human literature of an immunologic defect in any of these kids. Not just MPS VII but virtually all of the lysosomal storage diseases.

But I think that is more the fact that people had not looked because if you read their clinical reports there is always anecdotal reports about these kids get more, you know, they are more susceptible to pneumonia, more infections. But clearly the MPS VII mouse does have a subtle immunologic defect, which we believe is caused by defective or incorrect antigen processing through the lysosome.

Interestingly, though, we showed in that same study that that immunologic defect could be corrected in vivo in whole animal models following enzyme replacement. So it is a relatively subtle immunologic defect and at least by enzyme replacement it can be corrected. Now is it corrected in our AAV treated mice? We actually tried to do that experiment and for technical reasons the experiment just did not work but, unfortunately, you have to sacrifice the mice to do that experiment.

So I cannot definitively tell you that the immunologic defect was corrected in those animals but there is this inherent defect. Does it predispose them to tumor formation? It is hard to tell because the animals do not live long enough but that is certainly a possibility.

DR. BREAKEFIELD: We will take four more questions. Actually Kathy High has been waiting very patiently. I did not even see her there and then we will do Terry and then we will do two over there and that is it.

DR. HIGH: Okay. Mark, I just wanted to point out that one of the differences between treating MPS VII with bone marrow transplantation versus with an AAV vector approach is that in the bone marrow transplanted animals the source of beta glucuronidase is principally from circulating hematopoietic cells, some of which eventually become fixed. Whereas in the vector-based approach there will be pockets of transduced cells that make very high concentrations of beta glucuronidase. Do you think that this may have any relationship to what you are seeing?

DR. SANDS: It certainly could, Kathy. I mean, the data I presented -- the comparisons really are apples and oranges but it is all that we have got to go on right now and the reason I presented all that data is just to really highlight why we were so concerned about this finding because we have just never seen it before.

But again, you know, enzyme replacement, when you do those experiments, you have this huge bolus of activity and you have these huge peaks and valleys, which is not what we see with AAV and bone marrow transplantation is different also. They are very different studies. It is hard to draw any -- we cannot draw any real conclusions from it except to say that what we saw in the AAV treated mice is different from anything we have ever seen before.

DR. BREAKEFIELD: Terry?

DR. FLOTTE: The last two questions anticipated mine to some degree, which is really to again bring up this point of nonuniformity of the gene delivery and I thought it might be interesting to -- if you could summarize for us what data you have regarding whether there are whole areas or whole regions of the liver that might perhaps not be -- might not have received the transgenes or whether those might correlate with tumors or vice versa.

And then the other issue of nonuniformity is the cell type and it was really -- my thinking in asking that question related again to this antigen processing defect because I would presume that you might more uniformly in bone marrow transplant or also in the transgenic have kupffer cells that are more uniformly expressing the transgene and might be more immune competent.

DR. SANDS: Sure. To get to your first question, the only way I can answer that is when we sacrifice the animals and look for expression, for example in the liver, we take sections of liver from different lobes of these animals and section and look for beta glucuronidase activity in situ. So far we have not seen any differences in distribution of enzyme. There does not appear to be any localized over expression except within individual cells and localized over expression or no expression in the liver. It does not matter what lobe we take, it looks pretty uniform.

With respect to your last question, it is certainly a possibility that since we are transducing -- and it appears as though we are transducing primarily hepatocytes and, in fact, the kupffer cells being, if you will, perhaps antigen presenting cells or some sort of immune type cell that perhaps there are some that escape correction or whatever.

Now the only thing I have to go -- the only way I can answer that question for you is by histopathologic evidence. When we look at sections from different parts of these livers we do not see any disease in any of the kupffer cells. That is not to say that a few of them do not escape correction. Again correction is measured here by the presence or absence of distended lysosomes. That does not necessarily mean that they are fully corrected.

There may be molecular defects there that we just cannot detect which may predispose them to tumors. I do not know that but at least by the criteria that we can measure there does not appear to be any evidence of disease in any of the immune type cells.

DR. BREAKEFIELD: I think we can only take two more questions from the floor and then we will -- hopefully in the discussion later we will --

DR. FRIEDMANN: Ted Friedmann. I am a member of the RAC. I have two questions.

One, did you say that one of the angiosarcomas was uterine in origin?

DR. SANDS: Yes.

DR. FRIEDMANN: Where was the other one?

DR. SANDS: The other one was actually sitting right at the base of the brain. It was not attached to the brain.

DR. FRIEDMANN: So you cannot infer anything from the origin of the angiosarcomas?

DR. SANDS: No, not really and again there were only two animals. But very different locations.

DR. FRIEDMANN: Yes. Number two, your quantification or the general studies quantitating the number of AAV genomes in tumors were in normal tissue. How -- do they depend on the assumption that the entire AAV genome is present or are you assuming also that small fragments of AAV can be integrated?

DR. SANDS: Well, first of all, the real time PCR data says nothing about integration but this -- the real time PCR, the PCR product is only from the promoter region -- the enhancer and promoter region of the cassette. We have no idea of what the structure of the genome is. It is just quantifying that small piece of the genome. It could be rearranged. It could be autosomal. It could be integrated. Who knows what it is.

DR. _____: Mark, did you say whether or not you did or could you treat non-MPS VII mice with the same preparation of AAV?

DR. SANDS: We did not. The reason we did not was strictly expense. At the time we did this there was a lot of animals and a lot of virus and it is expensive. So, you know, that would have been the best experiment and we wanted to do that but just for, you know, financial reasons or logistics we did not do that.

DR. _____: Does the preparation still exist?

DR. SANDS: No, it does not.

DR. BREAKEFIELD: Thank you very much, Mark. That was wonderful. I wanted to get a lot of questions. I think that is where most of the questions are in your data and how to interpret it.

So we are about an hour behind overall so I am hoping that the next speakers will be, you know, very succinct in presenting their information so we have as much time as possible for discussion later.

The next speaker is William Sly, who is one of the experts on lysosomal storage diseases.

## NATURAL HISTORY OF MPS VII DISEASE IN MICE

WILLIAM SLY, M.D.
ST. LOUISE UNIVERSITY SCHOOL OF MEDICINE

DR. SLY: Thank you for inviting me. My interests are somewhat tangential. I think we could maybe have those lights down and I could have that first slide.

(Slide.)

Thank you. As we said at the outset, every patient counts and this is the first patient with beta glucuronidase deficiency who was the prototype for the disease called MPS VII.

(Slide.)

This is Clifford at three years later. I do not show you many patients with this disease because the disease is relatively rare.

(Slide.)

This is the patient at 11 years of age and we followed him for -- we followed him until age 20 when he died suddenly of cardiorespiratory defect.

(Slide.)

Now the clinical features of MPS have been summarized by Mark. I will not dwell on them. Short stature, bony disease, coarse facies, big liver and spleen,

mental retardation, corneal opacities.

(Slide.)

Deficiency of beta glucuronidase is present in all cells and body fluids. There is enormous intralysosomal accumulation of gags, secondary elevations of other acid hydrolases, which turns out to be a good way to study them to study response. Excess excretion of urinary gags and abnormal granulocytes and leukocytes.

(Slide.)

Now as Mark said, the mouse turns out to be a perfect model for this disease. This patient has the intermediate form of this disease. There are more severe patients but the mouse has all the features that are shown by the patients and, in particular, the one we want to focus on is the life span. These patients -- the mouse -- the patients actually are quite variable. This patient, as I said, has the intermediate form of the disease. The most severe form of the disease presents as neonatal hydrops and the children actually die before birth. That is probably the commonest form of the disease.

The mouse with MPS VII typically has a shortened life span. It becomes deaf. It becomes blind. It eventually stops eating. The life span is about five months in the average. Ninety percent of them are dead by eight months and none of them live to be a year.

(Slide.)

The person who discovered this was Ed Birkenmeier who described it in the mid-1980s and tragically himself died of a brain tumor about a decade later.

(Slide.)

This was the original Birkenmeier mouse at 34 years of age or one of Ed's original mice showing you the dramatic shortening stature in its 34-year old or 34-week old sibling.

(Slide.)

So the first experiment that we did with Ed at that time when this was discovered was to see whether the human gene transgene could fully correct all the features of this disease because we were not sure at that time that all the features of the mouse disease were due to that deficiency and so we made a transgene by injecting the entire human -- we made a transgenic animal by injecting the entire 22 kb human transgene, bred that on to the MPS VII background, the heterozygote produced about 10 times normal levels of beta glucuronidase and the homozygote 20 times, and that mouse was absolutely normal, lived a normal life span, and was normal in every way that we could tell so we concluded that every feature of this animal's disease was related to the deficiency of beta glucuronidase and that the human gene could adequately correct it.

(Slide.)

Now since then many, many people have found this an attractive model for therapeutic strategies and there have been bone marrow transplant, enzyme replacement and gene therapy. I want to just focus on a couple of things in reference to this and this is a little bit redundant with what Mark said.

(Slide.)

We did a bone marrow transplantation study with Birkenmeier and Mark, a two year study. It was -- the efficacy was dramatic in that the life span was increased threefold, approaching that seen in normal mice. The spleen, liver, cornea, many things were corrected. The brain was not corrected, although the meninges and perivascular cells were corrected.

(Slide.)

Now the important thing I just want to focus on here, which is the effect on survival, this is the survival curve of the untreated mouse. This is the survival curve of the normal strain. The green line is the irradiated control mice and it shows the life shortening associated with irradiation and the lengthening here shows the improvement in life span associated with bone marrow transplant.

Now the important thing here is that these mice live to be -- as long as 700 days and in this total experiment there were 30 mice that were irradiated. There were four tumors in the irradiated group only. Three of them were in the controls and one of them was the tumor Mark mentioned in the MPS VII. So the MPS VII mouse at least in this experiment was certainly no more sensitive to tumors than the controls.

Subsequently we focused on enzyme replacement therapy and there were a series of experiments. We did neonatal enzyme replacement therapy and we showed that it could dramatically effect the course of the disease, particularly if it was started in the newborn. One could even get into brain. For the purposes of this conference what is more important is we did a set of experiments where we treated in the neonatal period for six weeks and then we stopped and then we studied how long the beneficial effect of that treatment would last and I think I have that here.

(Slide.)

And what you see here, this is the life span of the untreated mice. These are normal mice still living well beyond a year. Only one of those mice died in the course of that. All of the mice were sacrificed at the end of this period and none of these mice had any evidence of tumor. These were enzyme treated animals only in the first six weeks of life. Their life was prolonged by that exposure to enzyme just during the first six weeks of life to more than a year and none of them had any tumor at that point.

(Slide.)

Now I want to just tell you a tale of two different transgenes that are relevant, tangentially relevant to the theme of this conference. The first was a transgene that we made in order to make a tolerant mouse. Now you would say why is that important. Well, almost everybody that is doing enzyme replacement therapy and particularly gene therapy, everybody I know that tried to do gene therapy on this mouse, has used the human gene construct. Is that important? Okay.

So we wanted to find out if it was important and so we developed a tolerant mouse and first we found that the active site was E540. We identified the active site of the mutant. We made an inactive transgene and then we established that inactive transgene on a mouse -- on a B/6 background and then we crossed it on to the MPS VII background and we demonstrated that this mouse still had MPS VII. This human enzyme had no effect on the phenotype but we wanted to test the hypothesis that it would confer tolerance and whether tolerance is important.

(Slide.)

Now in order to make this we used a relatively weak promoter, the PGK promoter driving the human beta glucuronidase cDNA. We made the transgene and the mouse that was produced, the transgenic mice, now which is transgenic for a dead human enzyme, looks just like the MPS VII mouse that we started with. It has the skeletal features that are no different than the MPS -- typical MPS VII mouse. This is a control.

(Slide.)

It has all the lysosomal storage and here we have just a variety of

things to show you. The cornea, the retinal pigmented epithelial cells, the bones, the liver and the kidney.

(Slide.)

This mouse has MPS VII by all criteria. However, when we challenged this mouse with intraperitoneal injection of human beta glucuronidase it was dramatically different than the control. The control MPS VII mouse has a dramatic, as you can see here, this is an ELISA assay showing that the antibody titer in these four MPS VII mice goes out to $10^5$ or $10^6$. So these make a lot of antibody to human beta glucuronidase. A tolerant mouse by contrast did just what we wanted it to. It shows absolutely no response.

(Slide.)

Now you say who cares. Well, everybody who is treating the MPS VII mouse with the human transgene is, in fact, doing this kind of experiment and those mice are undoubtedly making a lot of antihuman beta glucuronidase antibody. And one has to ask themselves is this or is this not relevant to production of tumors or other side effects or the persistence if you are looking at persistence of expression or so on.

Or the question I raised to Mark, could, in fact, these antibodies select against retention of the expressing cells in the liver tumor and lead to negative results?

(Slide.)

Okay. So that is just -- I am now going to tell you the tale of another transgene.

We made another transgene and we used in this case -- well, I will skip that. The transgene that we wanted to make here was one that would express as much beta glucuronidase as we could possibly express. We were interested since everybody was doing -- interested in gene therapy, is how serious is over expression of beta glucuronidase.

(Slide.)

So in order to do this we used the transgene and this transgenic vector here is the same one that Mark used. Mark got it from us and we got it from Mia Saki. It was developed from Mia Saki as CMV beta actin globin entron and promoter, and I think it is SV40 but I am not sure, poly A. But anyway Mia Saki developed it for massive over production of secretory products in cultured cells. And we use it for that to make huge amounts of beta glucuronidase.

But in this case we made a transgene with just this construct and we had about ten founders and we found that they varied in their level of expression from 10 times normal levels in serum to 50 times normal levels to really sky high levels. We were interested in sky high levels so those were the only animals that were kept.

(Slide.)

Now I want to show you our massive levels of over expression. So these are serum levels of beta glucuronidase that one might find if you look at serum, heart, muscle and so on. These are the full increases that we see in our massive over expressors. Here this is not 2,000 units. This is now 2,000 times normal levels. 1,900 times levels. Heart, 8,337 times normal levels, 953. So these are two different levels. These are just two different counter lines showing these massive levels of over production of beta glucuronidase.

(Slide.)

Now we were kind of interested in whether -- what would be the pathology of these and they turn out to have not something you are interested in particularly but they have a beta glucuronidase storage disease. They make so much

beta glucuronidase that they make beta glucuronidase crystals which you can see in heart here.

(Slide.)

And in kidney we could actually see crystalline structures in renal tubules.

Now why is that relevant to this conference? Well, we noticed that as we grew those tumors that one of these tumor lines -- I mean, excuse me, as we grew these mice that one of these tumor -- one of these cells -- strains rather, developed tumors.

Now we were not studying tumor biology and we were not very interested in that so we put those aside and we put those aside and we focused on the other one. Interestingly, the one that looked like it was beginning to develop tumors developed -- was the lower level of those two producers, not the one that produced the higher. We were interested in the highest level producer so we focused on that one.

But prior to this conference I went back and I looked at the autopsy of animals that we had seen over the course of the -- over the course of time and --

(Slide.)

-- the tumor strain -- the tumor bearing strain, which we called WE18, the second one, all nine of the animals that died under our care had tumors as it turned out when they were autopsied. One had a cerebellar meningioma at four months. The other eight were autopsied between 15 and 23 months. Six of the eight had one or more mammary adenocarcinomas. Four of the eight had pituitary adenomas. Two of the eight had adenoma carcinomas of the Zymbal gland. And the Zymbal gland I learned since that pathology report is a small gland that is peculiar to rodents at the base of the ear and one of them had bilateral pheochromocytomas.

The WE strain -- now we did not -- we autopsied a number of those along the way. I only have one of those that was autopsied as late as 24 months and it had no tumor.

So when I heard that there were tumors that developed in the AAV treated animals, I -- can we have the lights, please, now? -- I just assumed that they were seeing something like we were seeing in our one transgenic line and it was probably integration site dependent.

Here we made ten trans -- I think we had ten -- eight or ten founders. I cannot remember the exact number. But of those, one of those was a massive over producer of beta glucuronidase and it develops tumor in almost every animal that lives two years. I think -- personally I have assumed that that is because it integrates because it is this peculiar combination of beta actin and CMV promoter, which as you can see can produce enormous levels of over production of the enzyme, and that may or may not be related to the AAV phenomenon but I think it is interesting.

(Applause.)

DR. BREAKEFIELD: Okay. We are only going to take two questions. You will have to fight over them. Mark was up first.

DR. KAY: Randy and I were discussing the actual fold elevation in the liver was actually quite low. It was only seven-fold and with AAV in a transduced cell I would expect you would get more.

DR. SLY: Yes.

DR. KAY: So my question is in the --

DR. SLY: Interesting. Let me just amplify on that. When -- this is not unique to us but when these animals were studied early, the expression in liver was

quite a bit higher. It was fairly dramatic and then as has been observed with the CMV enhancer in other studies it dropped off. It is still at seven-fold now.

DR. KAY: So I guess my question is, is there any correlation with the tissues that you see the tumor and the fold elevation and enzyme production?

DR. SKY: Unfortunately, we do not have that data because we were not studying that at this time and we just went back and found these. We can do that prospectively but we do not have it. We have no data on breast, which is where most of these -- many of these tumors were found.

Yes?

DR. CRAWFORD: Coming at the question from a slightly different angle, on the one hand the bone marrow transplant correction is primarily that of fixed macrophages, whereas over expression such as you are discussing is in the epithelial and parenchymal cells of these target organs. Do you have any insights in tumorigenesis or, you know, different disease model between these sites of expression?

DR. SLY: No, I really do not. I think, though, that -- I do not have any on that but I should anticipate Randy's thing a little bit and say remember what I showed you was that the largest level of over expression did not develop tumor. So I do not think it is over expression of beta glucuronidase competing for the Man-6-P receptor that is responsible for this. I think it is probably another explanation. I do not have any other thoughts on mechanism.

DR. BREAKEFIELD: Thank you. I think we will move on -- we are hoping for a lot more discussion later but we are trying to keep the discussion to the roundtable discussion. So we are going to have another interesting talk now by Randy Jirtle and the last talk was fascinating and very succinct so, hopefully, we will see another repeat of that.

DR. JIRTLE: This is going to be fascinating but not --

DR. BREAKEFIELD: I have the opposite.

## B-GLUCURONIDASE BINDING TO THE MAN-6-P RECEPTOR AND EFFECTS ON IGF II BINDING

RANDY JIRTLE, Ph.D.
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DR. JIRTLE: When I was reading over the brochure this morning about what was going to be presented, Bob Maronpot, you are going to have to tolerate this story one more time, but it was like deja vu all over again. Ten years ago Bob and I were at an ILSI conference and it was entitled "Mouse Liver Tumors Symposium."

And we broke at lunch and on the way on up -- I had to go up to my room, I went into the elevator. An average Mr. and Mrs. American Citizen got on the elevator with me and usually -- I am sure I am like most of you, I went to the back of the elevator and they were in the front, and I always look at the numbers trying to think about how long I am going to have to stay in this place because I do not like being in tight places with people.

And I felt this stare and the guy was looking right here at my name tag. And he looked at it and he had this incredible disbelieving look on his face. He looked down at me and he looked down at him. And he said, "Mouse liver tumors? Is that a problem?"

(Laughter.)

Now you could imagine we had a whole symposium on this. So I was

so taken aback, the only thing I could say, "It is if you are a mouse."

(Laughter.)

And now I am going to start my lecture.

(Slide.)

I am going to focus my talk mainly actually on the phenomenon of genomic imprinting and hopefully by the time we get to the end of this you will understand looking at what I call the Man-6 phosphate IGF II receptor how this links together with what we are talking about today.

(Slide.)

Now if any of you have watched "Walking with Dinosaurs" or "Jurassic Park," you think that the biggest competition that occurred back in the Jurassic era was between T-rex and his fellow dinosaurs, which he liked to often have for lunch but I want to point out from our standpoint actually the biggest battle was occurring in these very, very small rodent-like sort of probably marsupial animals and it was a genetic battle between the sexes that ultimately gave rise to imprinted genes.

(Slide.)

Now imprinted genes are very interesting. We guesstimate that there is probably about one percent of the genome, which now is around 300 genes, are imprinted. And the definition is "imprinting is an epigenetic chromosomal modification that results in parent of origin, monoallelic expression." So, in other words, even though we inherit one copy of autosomal genes, one from the mother and one from the father, there is a subset of genes in which only one copy is functional and depending on the gene it is either expressed from the mother or from the father.

(Slide.)

Now these imprinted genes have many significant biological consequences. First of all, many of them are involved in embryogenesis. IGF II is a very potent growth factor. If you knock it out, the animals are very small, about half size. The Man-6-phosphate IGF II receptor is also imprinted as I will show and when you knock this out the animals are very large and it is actually lethal.

(Slide.)

Interestingly, these genes also give rise to many behavioral developmental diseases. These are pretty clear when you are talking about disorders like Prader Willi Syndrome and Beckwith-Wiedemann Syndrome but also there are very subtle differences. PEG1/MEST and PEG3 when they were knocked out in humans actually gave rise to the phenotype where the mothers abandoned their offspring so they are nurturing genes. These genes now have been found to also be imprinted in humans. Interestingly, the functional copy is not inherited from the mother but, in fact, is inherited from the father. And many of these genes are involved in carcinogenesis.

(Slide.)

Now the first evidence that imprinted genes existed came from some very fundamental and incredibly interesting, I think, studies that occurred during the mid '80s and that was from McGrath and Solter and Serrani and his colleagues where they were doing interesting transplantation studies where they would put pronuclei back into enucleated cells. If they put male pronucleus in a female back into the egg they would find everything worked fine. If, however, they took two female pronuclei and put in they found that they had a reasonably well developed embryo that was small that had virtually no placenta and the reason why this embryo is very small is it is hard to support embryonic growth with no placenta.

Now on the other hand if you take chromosomes or pronuclei that are only from the male you find there is virtually no embryo but, in fact, there is a very large placenta so it is very obvious that there is a functional difference between the maternal and the paternal chromosomes that we inherit and that genomic imprinting is the reason why we do not have carcinogenesis in mammals.

(Slide.)

Now obviously if any of you remember your genetics, which I do not remember it very well, if you paraphrase Mendel's second law, the parent of origin of an allele should not affect its expression. So, for example, if you look at this example up here, you have a maternal allele and a paternal allele, you put them together and you have a normal developing embryo. This X shows that this allele or gene here is inactivated by some point mutation or deletion but since the one that is inherited from the father is still expressed, you have a normal functioning embryo. Let's say these are very important in embryogenesis.

However, if you look at an imprinted gene you can see that this thing changes very dramatically and that is now you have -- here I have a stop. In other words, the allele you inherit in this example from your father is not expressed ever in any tissue but the one you inherit from your mother is expressed in the tissues. Normally this is fine and you get a normal embryo. However, if you mutate now the maternal allele you can see that you now have no functional copy of this gene at all and you now have in this example an abnormal embryonic development.

So, in effect, what imprinting has done is blocked the protection that diploiding provides us from recessive mutations.

(Slide.)

Now you have to say, "Boy, there must be some darn good reason why evolution would have selected for these genes?" But, in fact, the main theory for why these genes evolved says that there is no advantage of this whatsoever and that it is mainly a consequence of a genetic battle between the sexes to control the amount of nutrients that the offspring extract from the mother.

To put it another way, if, for example, you think of the ability, and a male is only going to mate with a female once -- we are talking about rodents now, not us -- it is to his advantage to have that female put as much nutrients as possible into that offspring so that it becomes -- and it is large so that it has a better chance to grow and develop and out compete other offspring.

But from the female's standpoint this is a very major disadvantage because she has to pass her genes forward completely by herself in effect. Only within herself can she pass the genetic information in her body forward. So there always has to be a reserve for the female to potentially mate with other males to pass the genetic information forward. Whereas, with males they can go from female to female to female to female. So you can see now you have developed a conflict which natural selection can work on and this then is proposed to be the reason why these imprinted genes ultimately are developed.

(Slide.)

And, in fact, you would predict with this model that genes that are paternally expressed -- in other words, the mother shut off her copy -- would be pro-growth and genes that are expressed from the maternal allele -- in other words, the father turned off his copy -- would be anti-growth and thus far all the genes that are imprinted that have been looked at, these predictions hold.

The other prediction was that imprinting evolved with the evolution of

eutherian mammals. The reason for this is because you can hardly think of a better battleground for extraction of nutrients from the mother than with the advent of the invasive presentation and large degree of fetal development within that offspring that occurs within-side the uterus.

(Slide.)

We wanted to test that hypothesis. And we decided then to look at the evolution of imprinting.

(Slide.)

Now the way we do this is look at what we basically have been provided with as far as the three main groups of mammals that are present and still here on earth. There are three. One is the monotremes and there are two members of that. The platypus and the akidna, and these are egg laying relatives. Marsupials, of which we only one member of that present in North America. Most of them are in Australia. And that is the opossum. Down South we call them "Road Kill."

(Laughter.)

And eutherian mammals of which we are part of. Rodents, cows, et cetera, are all part of the eutherian mammals. These are the true placental mammals. And you can see approximately when these things branched off. About 150 million years ago monotremes branched off and about 100 million years ago marsupials branched off and eutherians.

(Slide.)

Now we decided to use -- the gene we wanted to study initially was the Man-6-phosphate IGF II receptor. The reason we were interested in this is because, as I will state later on, we had demonstrated and now numerous other people have shown this, also, that this functions as a tumor suppressor. And it also has very interesting biology. I am surprised nobody has actually talked about it yet so I have to actually do some of this.

But mainly 90 percent of the receptor sits inside the cell where it functions like a shuttle craft bringing lysosomal enzymes from the golgi apparatus to the lysosomes but there is another little cycle that occurs between them. It is also in the membrane, between the membrane bound and also the lysosomes.

This gene or receptor when it sits out on the surface functions basically to control the bioavailability of growth factors that are very important in carcinogenesis. Mainly IGF II and any glycoprotein that has Man-6-phosphate tags on the sugars.

Now this is a little wrong because apparently this forms a homodimer so that rather than these M-6-P proteins binding within one single molecule, they actually bind across two but nevertheless the same thing holds. And there is an independent binding site for insulin-like growth factor II and it is not a signalling pathway. This is a degradation pathway. The signalling goes to the IGF I receptor and also the insulin Type A receptor.

This receptor is also involved very much in the activation of a potent growth inhibitor, mainly TGF beta. The latent protein binds on to this receptor, plasminogen, which is also bound here clips it, and the TGF beta is then released, and then it can inhibit cells from growing.

So if you have low levels or no levels of this receptor you cannot appropriately inactivate IGF II, which is a potent growth factor antiapoptotic factor, and you also on the other hand cannot very readily activate TGF beta, which is a very potent growth inhibitor proapoptotic pathway.

So this is a very critical gene in the controlling of the bioavailabiltiy of these proteins and probably others that we do not know.

(Slide.)

Now to look at imprinting, and I will go through this very briefly. First of all, we looked at platypus and we looked at opossum. Interestingly, the platypus IGF II receptor should not be called that because it does not bind IGF II, which we found. That evolution actually occurred somewhere between monotremes and marsupials. Marsupial M-6-P IGF II receptor does bind IGF II and everything up above. Whereas monotremes, chickens, et cetera, anything that has been looked at down below, does not bind IGF II.

So when we looked at imprinting we actually did not expect to find this gene to be imprinted because it would not be able to control the bioavailabiltiy of IGF II anyway and, in fact, that is what we did find. Just like in the chicken when you have a polymorphism here at the DNA level you can see at the RNA level at various tissues both alleles are expressed. This is a nonimprinted gene in monotremes.

However, when you go to marsupials, in this case the opossum, again you have a polymorphism sitting here, this is the DNA level, you can clearly see in this example the T allele, there is not a doublet here, is missing. The marsupials are imprinted even though they do not have invasive placentation. So that phenomenon of having fetal growth in an extended stage of that is not required to imprint genes and we also know now that the IGF II is also not imprinted in monotremes but is imprinted in marsupials.

(Slide.)

Now there is a lot of talk here and why is this important? We then looked at the whole family tree basically looking at the M-6-P IGF IIR and also IGF II. And, as you can see, if you follow this, this is IGF II receptor, the M-6-P IGF II receptor. Imprinting evolved at this locus approximately 150 million years ago with the advent and probably a precursor to marsupials and eutherians. Monotremes are not imprinted at this locus.

But, interestingly, as you go up the family tree, imprinting at this locus is lost. So imprinting is divergent at this locus in mammals. This is the only example of this. IGF II does not have this phenomenon. Once it is imprinted it remains imprinted all the way up the family tree.

Now why is this important? By the way, this is the very first gene therapy because you have an allele that was turned off but by some mechanism nature turned that back on. So 70 million years ago we now know gene therapy was at work. We do not know what happened here but we are not the first to invent it.

So why is this important, though? Because rodents, which are mice and rats, have only one functional copy of this gene. Whereas, we, as humans and everything above bats actually, have two functional copies. So some of you are probably saying why did we invite this person because all I have heard is what I have heard in zoo 101 for the last ten minutes?

(Slide.)

And here is the take home point. The IGF II receptor is a tumor suppressor gene. It is inactivated and is involved in every cancer that has been looked at, which includes liver, breast, head and neck, lung. It is an oncogenic target for mismatch repair because it is a poly-g region in colon and gastric cancers. So there is a large variety of tumors. In liver cancer, which I will show on the next slide, 60 to 70 percent of hepatocellular carcinomas in humans have this gene inactivated, at least one

copy. And in every other one it ranges somewhere between 30 and 70. So in reality this receptor as far as its breadth and extent rivals p53 as a tumor suppressor gene.

(Slide.)

Interestingly, which came out in Nature Genetics not very long ago, epigenetic changes, which actually control the expression of the IGF II receptor, have been shown to be involved with what is called fetal overgrowth syndrome in cloned sheep. Which means that if you get through the first stages and the embryo is developing with cloned animals now, you have a real problem way at the end. They either die just before birth or just after birth and that is because they have lost in effect the expression of the IGF II receptor because of an epigenetic change that turned off an internal allele, and these animals are about 50 percent larger than normal.

We now do not have imprinting at this locus so it suggests that it may -- if this is a main problem with cloning at least at the later stages, it might be actually easier to clone humans than it is to clone a cow or a rat.

(Slide.)

Now another interesting thing is that Granzyme B, which is released from cytotoxic T cells, is targeted into the cell that is supposed to be killed by the IGF II receptor. So if you do not have high levels of this receptor present, you, in effect, are protecting that cell from immunological surveillance.

Finally, just a little thing nobody has ever done any more on it, it has actually been identified as the first putative IQ gene. I say that only smart people must work on this receptor.

(Laughter.)

(Slide.)

Now getting back to liver cancer which is what we have heard a lot about here. This is a human hepatocellular carcinoma in a cirrhotic patient. I do not remember if this patient was chronically infected with hepatitis B or C but it makes no difference. The results are the same.

And a very interesting -- this is work that was done by Tomoya Yamata. And I must say that the person that did all the evolution work just passed his Ph.D. as part of his M.D./Ph.D. last Friday and his name is Keith Gilliam.

Anyway, when Tomoya Yamata looked at this, he was scraping out these areas and you can find -- see there is a polymorphism that we used, and we found that one allele was lost in all of these areas. And I think this tumor also had the other allele mutated but I do not remember for sure.

But the interesting part is when you went into these areas outside of this tumor, here, here, here, here, the hatched areas, and lo and behold we found also that one allele was lost here. These faint bands are because of contaminating normal. If you look at 12 you see the bottom allele is lost.

What this suggests now is that this whole volume of tissue is clonal. It appears that loss -- and it is not mutated. Whenever we found the tumor had the second allele mutated, the surrounded normal tissue that was right adjacent to it did not have that mutation. So it appears at least in these tumors the first hit is loss of one allele. It apparently gives it a growth and/or survival advantage in this chronically infected environment and these cells literally regenerate that liver.

Now if you think of it from the standpoint of the IGF II receptor, this tissue right here is genetically identical to the whole liver in a mouse because the whole liver in a mouse only has one allele of this gene also functional because of

imprinting.

Seventy percent of hepatocellular tumors come out of cells that have the IGF II receptor, at least initial cell, one copy inactivated. Only 30 percent come out of these cells over here that have both alleles present.

(Slide.)

So what we are proposing as a model is that you have these cells in human liver for what unknown reason have at least one copy of the IGF II receptor already inactivated. Now it does not mean that there could not be other genes that do the same thing but when you get this chronic infection where you are losing cells all the time, you also have to have regeneration to actually keep this mass at about the same volume as you always -- that you had initially. So I have this necrosis here. It does not look like that but, you know, that area is what I say is necrotic.

Now where you have these cells that already have one allele lost of the IGF II receptor you get clonality of cells that are phenotypically normal. If you look at these cells with just H&E they do not look abnormal. They are in an abnormal environment but they are not abnormal as far as having physical appearance. And ultimately, as I said, 60 to 70 percent of the tumors come out of these clonal masses that have only one copy of the IGF II receptor functional, not out of this tissue here that has both alleles functional.

(Slide.)

So what we are saying is that this provides an explanation possibly, at least one, of why there is a species dependent difference in risk basically of mice and rats to cancer relative to humans. Now if this is the case given the fact that this receptor is involved in trafficking lysosomal enzymes, if you do produce high levels of these enzymes either intracellularly or even outside of the cell, and you, in effect, reduce the level of receptors that are present on the outside of the cell, this could potentially have an oncogenic effect itself. I am not saying that what you see here is this but if you are really jacking these things up locally very high, you could create an environment when, in effect, you have knocked out both alleles of this tumor suppressor gene in mice.

If this is the case, however, this would probably be less problematic in humans because we have both copies of this gene functional unless you are trying to treat patients that have chronic diseases where you would get regeneration of the liver in which you might ultimately get cells that regenerate that liver having only one copy of this tumor suppressor gene functional.

So if you think about it, what acutely caused that patient to survive from fulminate hepatitis basically -- in other words, these cells in this environment have a growth advantage, ultimately gives rise to their demise because you are regenerating your liver with preneoplastic cells.

(Slide.)

Then we also wanted to ask the question, for example, if these cells are more resistant to dying in their normal environment, are they potentially also more difficult to kill as far as when they form a tumor. So just to be focused on this yellow versus the blue, this is for head and neck cancer now, not liver cancer, and this is a Phase III study.

This was not part of it -- the original part was we were comparing chemotherapy alone -- radiation therapy alone plus combined modality and you can clearly see, and it was highly significant, that we do not have the receptor mutated, which is non-LOH, we have very high survival. Whereas, when it is mutated you

have very low survival and it appears from this retrospective study anyway that patients that benefit most from chemotherapy are the ones that have the IGF II receptor mutated. They go from here to here whereas this line goes from here to here.

(Slide.)

Now I think this is kind of interesting. Not only do these cells apparently appear to be more resistant to death, et cetera, when they are in their environment in the liver, for example, but they also are more resistant apparently when you try to treat them after a bona fide tumor has resulted. If you remember in head and neck cancer there is things called the field effect where there are multiple primary tumors that develop within an area. More than likely you are getting clonality again of these preneoplastic cells that look phenotypically normal that are also already premalignant.

(Slide.)

This is a phenomenon that is going throughout the cancer world --

DR. BREAKEFIELD: Hopefully, we are going to try to start summing up here?

DR. JIRTLE: Yes.

DR. BREAKEFIELD: Okay.

DR. JIRTLE: I just wanted to stop here then with the fact that we have now made a fluxed M-6-P IGF II/R mouse and with this because this is a lethal mutation when you knock out embryonically, we have fluxed the exon 10 and as a consequence in this case you can see we have now bred this with a Cre animal that produces Cre in response to albumin and you can knock the gene out in the liver.

So I do not know if this animal would be of use to what we are talking about here. It surely is of use to me because I want to determine whether or not these animals now are more sensitive to liver carcinogenesis in general but they might also be of help to resolve some of these issues that have been discussed this morning.

Thank you.

(Applause.)

DR. CRAWFORD: Randy, before you leave the microphone, a quick question. Could you clarify, when you say "trafficking of a lysosomal enzyme might impair things," are you proposing that as a second hit? If you have too much lysosomal enzyme to traffic it actually will functionally shut down the second allele?

DR. JIRTLE: You should ask Dr. Sly about this but it seems like one -- if you have high levels you could, I think anyway, alter the distribution of the receptor from being -- going to the surface to primarily, let's say, being in the inside if that is the case. Let's say that --

DR. CRAWFORD: And hence losing its tumor suppression function?

DR. JIRTLE: Right. If the kupffer cells are making high levels -- let's say it is coming in from the outside, now that receptor is going -- that enzyme is going to be competing with TGF beta for activation and IGF II so there will not be as many receptors there.

DR. CRAWFORD: But I think part of the question is how high is high because if it is kupffer cell --

DR. JIRTLE: I have no idea.

DR. CRAWFORD: -- uniform as opposed to transduced hepatocyte locally, those may be two different --

DR. JIRTLE: I think the local probably is going to be -- would probably -- if this is -- if this is the model it would have to be a lot higher than what

we have seen locally.

DR. KAY: The angelman gene missed being identified for many years because people were looking for an imprinted gene and it was only imprinted in a very specific tissue. Since you only have 30 percent loss of heterozygosity in the liver, do you know for a fact that in the human that this gene really is not imprinted? I mean, has that been looked at molecularly? Do you have a preference for loss of the maternal versus paternal allele? Have you used this polymorphism to look at transcripts and things like that in the liver?

DR. JIRTLE: Yes. In fetal and in adult stages it is not imprinted.

DR. KAY: Okay.

DR. JIRTLE: It is not just the evolutionary approaches. We have physically gone and looked at it.

ROUNDTABLE DISCUSSION

DR. JOHNSON: Any other questions for Randy?

Our next speaker fell prey to the MacIntosh curse, I think, and is having trouble getting his slides set up so what we are going to do is we move right on into the panel discussion at this point. It makes sense to do that given the agenda that we have to discuss and I think that -- I believe that this was included.

The panel discussion questions were included in all the folders. And while we are getting started here, Amy is going to put up the questions on the slide.

So I think what we will do is just simply ask the questions and see if the roundtable panel here at the table has an answer that they would like to pose for the question. I want to try and stick to these as much as we can but then also leave room for some extraneous discussion because I have a couple of points that I would like to raise as we get into this.

So the first question is: "Please discuss the potential for the AAV vector system to induce tumorigenesis: (A) What is the potential for AAV integration to induce insertional mutagenesis?"

Does anybody want to tackle that on the panel?

Nick?

DR. MUZYCZKA: Well, there is always a potential it will cause insertional mutagenesis. Any time you put any kind of a DNA into a cell that does not belong there, whether you do it free of plasmid or whether you do it via an AAV vector or a lenti vector, or whatever, if it is sitting there for a very long period of time we all know that mammalian cells are quite proficient at illegitimate recombination and so there is the potential at least for illegitimate recombination and insertional mutagenesis.

Having said that, if you look at the data on these specific tumors, I think it is fairly clear with maybe one minor caveat that this is not a classic case of insertional mutagenesis for several reasons. One is we cannot find the DNA for all intents and purposes in the tumors. And when we do find it, it is down at a very low copy number as compared to the genome. And that, in fact, might be our routine test. We are going to run into this problem all the time and we might consider using real time PCR and just looking at the ratio of the gene that we put in, the vector genome copy compared to the cellular genome copy, that ratio as an indication of whether we are looking at insertional mutagenesis or not.

If it is well below one, which it is in all these tumors, then it is unlikely that what we are looking at is a clonal expansion of an insertion into a tumor suppressor gene. That does not rule out other kinds of tumor induction models and we

have heard some of them but that one at least seems to be ruled out in this particular set of tumors.

DR. JOHNSON: Any other -- I think that is an elegant answer that summarizes the data very well.

Are there any other points to be made in response to that particular question?

DR. BREAKEFIELD: I would just like to ask a couple of questions on that. One is let's say if you just take cells in culture, a human cell that is dividing and you infect with a vector that is rep-, what is the frequency of integration in the genome or do we know that at this point?

DR. MUZYCZKA: That is fairly old data. Again from cell culture -- and I want to caution everybody that what we learned about AAV integration in cell culture appears to have no relationship to what we are learning now about AAV persistence in real animals. But the data is variable with cell type. In general, rep+ clones were integrating, we now know, predominantly into chromosome 19 at a maximum frequency of about 10 percent.

And so-called recombinant rep- clones were integrating anywhere from .1 to 80 percent depending again the cell type. And that is a real 80 percent, i.e. if you put 80 -- 100 cells on a dish you got 80 of them transduced.

There have been arguments about those numbers but that is what we have been seeing. I think that is a fair description.

DR. BREAKEFIELD: Is that transduction -- do we know whether it is integration?

DR. MUZYCZKA: Yes. In those cases in the cell culture experiments we generally were using selection, which some people would argue is a confounding issue, but even when the selection was not there -- for example, there was a set of experiments done with wild type genomes in cell culture where you -- there was no apparent selection. You simply cloned single cell clones and that has been done with some vector as well. And, yes, those numbers hold up.

DR. BREAKEFIELD: And I just have one more question. If you do not have rep present and now you have all these integrins, have enough junctional interfaces with the genome been analyzed to say whether there is any kind of site preference to a rep- integration?

DR. MUZYCZKA: I am going to punt to Samulski on that one. He has probably done more of them anyone else.

DR. SAMULSKI: I think all of our data says there is no site preference for integration once you lose the rep chain. At the same time the type of integration is similar to what Michael Lenin (sic) and Robert described. They are very -- one or two copies that seem to integrate unlike the wild type, which can be high copy number.

DJ : That is in cultured cells?

DR. SAMULSKI: Yes.

DR. BREAKEFIELD: I just have one more question so I am clear on everything. Is the IGR when it integrates in itself, can that act as a promoter or -- I know obviously people bring in other promoters but is the ITR element really a promoter in its own right or, if so, how strong?

DR. FLOTTE: I just want to say the ITR promoter is -- has very weak promoter activity and it varies from tissue to tissue. It was discovered accidentally it is so weak. Looking at a functional readout of CFTR, which is where you actually do

not need very high expression in terms of messenger RNA copies per cell, when you use an ITR promoter in vivo, for instance, under conditions in a region along where you might get 20 to 50 percent of cells showing evidence of DNA, you get one copy of RNA per cell or less. So it is a very weak promoter.

Jude has recently shown that it can be high enough to be detected in the central nervous system when one is looking at baseline level but it is probably -- this is a less promoter and, you know, is extremely weak. No -- I mean, I would say probably if you wanted to quantify it, it -- the -- which has been done, the expression level was something like a luciferase or a cat reporter, it is probably at least three logs less than what we would consider a weak promoter like SV40 promoter and, you know, up from there compared with CB promoter. You know, five logs less or -- you know, I am just guessing but on that order of low efficiency.

I do not know if Jude wants to comment on that.

DR. JOHNSON: There was -- I think, Phyllis, did you have a question from the audience?

DR. GARDNER: Insertional mutagenesis certainly is not my field so I wanted to understand one point. Is it -- if you expected it to be insertional mutagenesis, and let's just hypothesize the Man-6-phosphate IGF II receptor, that leads to the failure in this imprinted species of the tumor suppressor gene, and then you looked at it to examine it to look for integration of that or -- of that DNA, you would expect a one to one ratio of the vector DNA to the genome DNA. Does that assume then that that is all clonal expansion of the -- is the tumor -- could it be that you start with that process and then the tumor has other mechanisms for growth than expansion? I do not know. I mean, is it expected to be clonal, the tumor?

DR. JOHNSON: I will get Nick to answer that.

DR. MUZYCZKA: The answer is yes.

DR. JOHNSON: Yes.

DR. BREAKEFIELD: I mean, it would be expected to clone but, I think, as Mark raised, some tumors, for instance, have a lot of vascularization associated with them so it does not necessarily mean that all the cells in there -- all the tumor cells are but there can be other cell types in there. Some tumors like NF1 tumors, I mean it is hard to find a real causative cell. There are so many other tumors that kind of join into the --

DR. GARDNER: What would you expect in the case of hepatocellular carcinoma or angiosarcoma?

DR. JIRTLE: Can I make a comment? I mean, when you lose function of the IGF II receptor you protect the cell from dying and apparently you also protect it from being killed by the immune system so now you have a perfectly wonderful cell for the accumulation of additional oncogenic events which will ultimately give rise to this thing growing very large and some of them will not but we do not see a lot of tumors probably in these animals but some of them will.

So you protect that cell from being destroyed and then it accumulates additional damage and now you have a tumor at a month or six months or a year later, and it is clonal.

DR. MUZYCZKA: Yes. But I think the -- but to go back to her question, if one of those events was due to an insertional mutagenesis event because it is clonal you would expect to see that there in that tumor.

DR. JIRTLE: That is correct.

DR. MUZYCZKA: Right.

DR. JOHNSON: One final point over here.

DR. _____: Yes. I just wanted to follow up on that question because if most of the cells in the specimen that are tested are, in fact, part of the tumor clone, which seems to underlie the assumption that you would see a DNA copy number of approximately one if there was insertional mutagenesis, then why would you not see a DNA copy number of zero? Why, in fact, are you seeing a DNA copy number of say .1 and that suggests either that you have a none -- that would suggest you do not have a clonal population, either the virus was present there or infected after the transforming event, or if 10 percent of the cells are infected and if that is higher than what you see in the liver, it could be that that is the number of malignant cells and that the other 90 percent of cells in the specimen are something else.

What explains that lower copy number?

DR. KAY: I mean, first you have to realize when those tumors are resected they have some normal tissue there so you are going to have some of the AAV from those cells as well that may be in the population.

DR. _____: So we should revise the earlier statement to say that if it were insertional mutagenesis we would expect not a copy number of one but a copy number greater than a certain amount so then the question is, is that number greater than .5, greater than .1, greater than .9, greater than .01? What percentage of the cells in that biopsy are tumor cells and can you find out which ones of the cells --

DR. KAY: It looked like it was a predominant cell type and the copy numbers, both by quantitative Southern blot and by real time PCR, were very concordant and these were totally done in separate facilities. We had no idea what the other person was doing and at the best -- at the very highest it was .1. So that is tenfold less than a number of one, a ratio of one.

DR. CRAWFORD: You can actually go back to the 1965 morphometric data which shows that in normal liver roughly 90 percent of the nuclei are hepatocytes but the remainder is everything else. It is sinusoidal (phonetic) endothelial cells, kupffer cells, fibroblasts and what have you. And although the proportion may be a little bit higher in a hepatocellular carcinoma you still have vasculature in sinusoidal cells to deal with and one could even make the argument that the copy number you are seeing is the endothelial cells and kupffer cells.

DR. FLOTTE: I need to clarify one point on our data, I think, which is that we examined DNA from four tumors. We had no detectable vector DNA in two of the four tumors and, in fact -- I mean, by my reasoning, if this was a necessary and sufficient event for tumor formation that we should have had results that were consistently within a range that would be at least, you know, feasible given the possibility of other kupffer cells.

DR. CRAWFORD: And that copy number arguably would be .9 or greater or something like that.

DR. JOHNSON: Right. Let's make two more points and then we have got to move on.

DR. KAY: I just want to make the point that there has been a lot of data generated clinically and preclinically with retroviruses, which I think are much more efficient at integration except in the rare -- the exception where there was a lot of wild type replication in monkeys, there has been no evidence of tumor formation to date.

DR. GARDNER: Just one more question. Insertional DNA, is it as stable through replication as the regular DNA? Is it ever lost once it is inserted and

rapidly turn over cells or does it persist?

DR. SAMULSKI: I think you bring up the one variable that may explain some of this data and that is if you had an integration event that caused a problem and then it got lost, and the tumor went on without the primary vector still present, that model has been referred to as a hit and run, and whether or not it is something that has an impact here, I do not think any of our data can support.

DR. FLOTTE: The point I just want to make in terms of organizing our thinking about this, I mean we have thought that generally in animal tumorigenesis that insertional activation is a lot -- is a lot easier type of a model to say it would happen with some predictable frequency. With the exception of genes that are imprinted, most tumor suppressors would have bi-allelic expression and so one would have to have the vanishingly rare event of two hits on separate chromosomes.

So a hit and run is not going to be an insertional activation event. More likely that would be an insertional deactivation event and that -- so that would only apply to a very small subset of tumors. I think this issue about the imprint allele is important in terms of the transgene product in this particular instance but to think generally of tumor suppressors as having monoallelic expression is not -- is leading us in the wrong path.

DR. JIRTLE: There is presently only two tumor suppressors that are known to be imprinted and only one in humans.

DR. BREAKEFIELD: Could I just make a comment about the retroviruses? I mean, usually they activate a protooncogene downstream.

And also the one thing I was kind of curious on that, with retroviruses they have the advantage that they are pretty unstable usually and titers are pretty low so in contrast to AAV you can get -- they are more stable and you get, you know, higher titers. So just in terms of the number of hits the cell takes what is the relative --

DR. KAY: Well, with newer vector preparations you can get pretty high titers and we have used lentivirus so we can transduce over 50 percent of hepatocytes and we know in those examples that if you do a hepatectomy you have 100 percent integration, at least of the expressed genomes. So in that situation, unlike AAV where there is a very small percentage of the cells that are transduced that actually have integrated genomes, with retroviruses it is close to 100 percent.

DR. JOHNSON: Okay. One more?

DR. SLY: Yes. I would like to say in reference to the hit and run two things. One is this has an enhancer that can have a very long-range effect in reference to activating protooncogenes at some distance.

Secondly, I am wondering whether the immune response could, in fact, encourage run once the activation has occurred because it is clearly evoking -- very likely evoking a very strong immune response to a cell now that is making a foreign gene product and it may, in fact, select against those cells which will be eliminated and lead to low count residual AAV.

DR. JOHNSON: I think we might address the immune part in number two because I have some questions.

A final word from the FDA?

DR. SIEGEL: Yes. Well, I am trying to put together these comments and I am not sure I understand so let me express what I understand. There were four mice with tumors and in those tumors the copy number was undetectable in two and was up to .1 -- was positive up to .1 in two of them. And now we are saying that maybe as many as 10 percent of the cells in these specimens were not from the tumor

clone.

So are we suggesting that in some mice 100 percent of the nontumor cells expressed the gene and in other mice zero percent of the nontumor cells express the gene or what is -- I am trying to get an understanding for the explanation where is the signal coming from, what does it mean that it is higher than in normal liver tissue if you are dealing with clonagenic cells?

Do we really know that 90 percent of these cells are tumor cells or could it be much less? In some tumors it is much less than that. Could they be mostly inflammatory cells or infiltrating cells? What would explain this?

DR. CRAWFORD: Number one, I do not think we have excluded a variety of possibilities so, I mean, that was simply a morphometric statement. I do not know where the gene is being expressed. If it is being expressed in hepatocytes you have to say that 10 percent or less are carrying copy. If it is expressed in nontumor cells then you have to address variability in those nontumor cells and call them inflammatory or stromal cells. So we have a variety of ways to interpret the data and I do not think currently we can distinguish.

DR. SIEGEL: Do half the cells express it or is there an experiment to --

DR. CRAWFORD: In situ would be a way to do it.

DR. KAY: We have done some -- I am sorry. We have done some of this. We have done immunohistochemistry. We have done X gal staining. We have done RNA in situ hybridization with a variety of different reporter genes. And we have not seen evidence of transduction of non-parenchymal cells. That does not mean it does not occur at a low frequency and in those experiments we have used ubiquitous promoters and we have looked for expression. So if something was being expressed in those tissues we would expect to see that. That does not mean that it does not happen at a low efficiency but we have not observed that yet and we have looked at a lot of different ways. I do not know if anyone else has done those types of experiments.

DR. SANDS: If I can make a comment real quick just to address your last point about the number of inflammatory cells in the tumors. When Carol Vogler, who is the pathologist who has examined most of these animals over the last 12 years, when she examined these tumors there is a very striking difference between a section of normal liver from the same animals and a section of tumor. The vast majority of these cells in the tumor are hepatocyte in nature but the vast majority of them are dysplastic in nature. There are also very few inflammatory looking cells.

So it really looks like a hepatic tumor is just a big aggregation of dysplastic hepatocytes with very few other cells in there. A few endothelial cells and a few lymphocytes but mostly hepatocytes.

DR. FLOTTE: Now if I can just draw back one more point again in terms of sort of summarizing and thinking about this. I mean, people have talked a lot about the promoter and downstream activation and so forth. There would be no explanation if this is purely a genetic alteration event for the great discrepancy that we see between animals in this model. I mean, it is an obvious point but I mean if we are focusing in on just the behavior of the vector it would have to be very specific to the strain in order to see such a discrepancy in the frequency of tumors between the different sets of data.

DR. JOHNSON: Okay. Let's address point B. "Could different methods of vector production contribute to the tumorigenic potential of the vector?"

DR. MUZYCZKA: Since I made it, of course. This particular vector,

which by the way was the same in both negative sets of data and the positive sets of data that you have heard about, was done by what is called the iodexonal step gradient density centrifugation followed by heparin column chromatography. So the major contaminants might be iodexonal or heparin. The other things that are in there are salts basically, the buffers that have a pretty good safety record. Iodexonal is actually, I believe, approved for human use as a contrast agent and we would have very small amounts of it. We have not gone to test for it. And heparin is also something that has been commonly used in animal experiments and again we would have only small amounts of it.

So I am hard pressed to see what in this particular virus might be a contaminant. Unfortunately we did not save as we are doing now, we did not save two years ago aliquots of this so we cannot go back and check for other obvious potential fortuitous agents like hepatitis C or hepatitis B. Again we did not see that in the companion set of data from other labs.

I think that that is why we do both acute and long-term tox studies to check for things like this that we cannot predict might be there and cannot predict might cause either acute toxicity or tumors but I have no reason to believe that this particular prep had any. So I think that is just something that is going to have to go on hold.

DR. SAMULSKI: Can I add a quick comment? I think if something coming through the prep was going to contribute to oncogenicity there is enough people out here making AAV that if there was a common reagent we would see more tumors showing up and I would also suggest that because of the proficiency in how virus is probably made for this study, if it was something that came through, it must be very, very specific because I think the quality is probably higher than what most of us do and, therefore, if there was a common agent we would all be facilitating this oncogenic event if that is where it is heading.

DR. FLOTTE: I had just one other question that is sort of prompted by this. Not directly related to production but the issue of contamination of the mice with other tumorigenic infectious agents such as helicobacter or viruses. I do not know if, Mark, you want to comment. You never had an opportunity to comment on that, whether, you know, there was the possibility -- since these were the only real long-term mice at that particular time.

DR. SANDS: Right. Very shortly after we discovered the tumors I contacted our animal care people and I got all the sentinel reports from our mouse room for the last three years. And during that time period there were no agents present in any of the sentinel animals. Now again these are -- it is a limited screen that they do. Actually it is quite a few pathogens they look for but obviously there are things that are missing and that is a viable hypothesis that something could have come in during that time window of time and contaminated those specific animals.

Just as an aside, I tend not to favor that only because in that same mouse room where we do all of our procedures and our mice are housed, we also have SCID MPS mice and non-SCID MPS mice, which are obviously very severely compromised. And I would predict that if there were some infectious agent in there that we probably would have wiped out those colonies and we have not seen any evidence of any toxicity of any kind in any of our colonies. Again it does not formally exclude that possibility but it is not one of my favorite hypotheses.

DR. JOHNSON: A question from the audience.

DR. DAWES: Roland Dawes, NIH. Is there a policy in place to hold

on to part of each lot used in a human clinical trial just in case something like this pops up and we want to go back and look?

DR. JOHNSON: Dr. Carter, would you like to respond to that?

DR. CARTER: (Not at microphone.) (Inaudible).

DR. JOHNSON: Yes is the answer.

DR. KAY: Can I make a comment about the adventitial agents? I have learned recently that in most mouse colonies they do not check for helicobacter and from what I understand in certain strains of mice the major phenotype of infection is after long-term -- some sort of hepatic tumors. And that is something now that I have asked our people to start checking for because from what they tell me, this is not commonly checked for in most mouse colonies.

DR. FLOTTE: Those lymphoid aggregates in the liver actually, I do not know if Jim wants to -- but those are commonly seen with helicobacter infection and that one finding that you did bring up.

DR. JOHNSON: Okay. Let's move to Point C. "Could co-infection with adenovirus or herpes viruses increase the oncogenicity of AAV vectors?"

DR. SAMULSKI: Can I take this one? I think it is very clear that once you make an AAV vector, in order to mobilize it you need two hits. You need an adenovirus or a herpes and you also need a wild type AAV to supply direct genes. Unless you have two hits, wherever the vector is, if it is integrated, it is not going to be mobilized so I do not think incidental infection where AAV is going to increase any probability of getting more oncogenic. I think once you do have two hits you have a lytic infection and then you are asking yourself what is the probability of vector moving, wild type moving and integration. And I just do not think anybody has addressed that question in any models where we could conclude any kind of information but I think vectors will not move.

And I think a number of people have looked at this where if you put -- if you have an integrated cell line or an integrated virus and put in helper virus it does not move.

DR. KAY: Some of the mouse data I showed about the first 10 or 12 mice were with very early preps and they were substantially contaminated with wild type AAV and we -- and those animals were followed 12 to 19 months and we did not see tumors but again the lack of adenovirus.

DR. JOHNSON: Terry, you have done an experiment where you have tried to induce mobilization?

DR. FLOTTE: Right. We did a series of three different paradigms of infection. In different sequence whether we would treat with vector. This was all in the respiratory tract and all in Rhesus monkeys, either infecting with vector and then subsequently co-infecting with wild type AAV and adenovirus. In which case we really did not see much in terms of mobilization of the vector or in some other different sequences.

The only situation where we saw some mobilization of vector was where we had co-infected at high -- at really high multiplicity of infection the wild type and the recombinant vector, and then subsequently seven to ten weeks later came back with the adenovirus and saw some very brief and low level shedding of the recombinant. It appears our hand waving explanation is that the wild type virus has a great competitive advantage when the helper virus comes along. And so where you need two hits, that two hit event with latent vector really tends to favor the replication of a wild type virus more than the recombinant.

DR. MUZYCZKA: I would just add one more point and that is if that event happened it would seem to me that now you are sending up a red flag to the immune system which is going to focus on the Ad but it is also going to clear eventually this -- Ad or herpes but it is going to clear that cell that is carrying the AAV. So this would be a mechanism really for getting rid of your vector rather than --

DR. FLOTTE: I would add on that as well. We did a related but different study where we could only see cell mediated immune responses to AAV. This was with wild type AAV in the presence of adenovirus. But when we did use adenovirus we saw both cell mediated and humoral responses to the AAV. So it was clearly in that scenario with productive infection where you really are getting -- you are sort of shedding the immune privilege.

DR. BREAKEFIELD: I think we can have our last talk by Robert -- our last talk of the morning by Robert Maronpot and he is going to talk about hepatic neoplasia in mice, which is I think some new information we would all like to have right now.

## HEPATIC NEOPLASIA IN MICE

ROBERT MARONPOT, D.V.M., M.P.H.
NIEHS

DR. MARONPOT: Thank you. Sorry about having a Mac although I like it for most things.

(Slide.)

I am from what we call NIH South. We are down in North Carolina and we also house what is known as the National Toxicology Program and boast, if it is worth boasting, over 500 cancer bioassays in rats and mice. So most of our experience is related to conventional animals but we are doing more and more work with transgenics.

(Slide.)

My initial idea was to give you an overview, and I probably should have talked a lot earlier. There are a variety of primary tumors that can occur in the liver. We are here interested in, I gather, mostly just the hepatocellular ones, although I was led to believe before I came that the hemangiomas or hemangiosarcomas were also hepatic but it appears that is not the case.

(Slide.)

I am going to focus on the hepatocellular tumors. I want to tell you that they are very common in mice. It is the most common response when we treat with a carcinogenic agent or a potential hepatic carcinogen.

(Slide.)

And in those situations where you are dealing with a chemical administration to see if you are going to produce tumors in rats or not, 24 percent of the time you get a positive liver response. So it is a nontrivial response. It occurs all the time and you see lots of liver tumors.

(Slide.)

Now there is a strain variability in terms of the spontaneous occurrence of liver tumors and there are high sensitive or susceptible strains and low ones. And not everything is on here because we do not have lots of data on some strains but I will point out that the C57 Black, which has been talked about here, is a low susceptible strain. So would be the FVB mouse if you are using that for your transgenic work.

(Slide.)

Well, we need to see what liver tumors are. This is your first exposure to something that you can probably identify as a liver tumor and there is two categories here. There is the spontaneous occasional occurrence of a discrete nodule in a liver that is tentorially different and is raised about the surface. And in more heroic treatments you can get multiplicity such that there are so many of these that occur that they begin to fuse and grow together.

(Slide.)

There is a progression of how these form and that is one of the main points I want to make. The progression goes from foci to cellular alteration to adenomas, to carcinomas. When we are talking about outcomes of studies, some of which we have heard today, we are looking at a window in time and we see what is present in that window. We do not necessarily see the progression.

(Slide.)

Foci of cellular alteration of the earliest lesions, they increase in number and later on as they transform into adenomas they decrease in number. Some adenomas are seen to arise within foci when you are lucky and you get that fortuitous histologic section and the adenomas increase in time before the carcinomas. Then the carcinomas come along and sometimes we can see those focally arising within adenomas. So a number of us, myself included, believe very strongly in this progression.

(Slide.)

This is the focus of cellular alteration. It is a tentorially different population of cells that does not compress the adjacent hepatic parenchyma.

(Slide.)

This is a little more subtle but it is a focus that is basophilic and this is all normal liver around the edges.

(Slide.)

Adenomas are more discrete and they are made up of a monomorphic population of cells. That is one example and here is another example.

(Slide.)

Now carcinomas are bigger, more heterogeneous. I do not have a low powered picture but they have a distinctive histomorphology on high power where the clusters of hepatocytes are arranged in nests or cords or sometimes glands, and these are rather thick trabeculae separating the sinusoidal spaces between them where these other cells, these non-parenchymal hepatic liver cells reside that we have just heard about in the discussion.

(Slide.)

I am going to tell you about an experiment to make a point because I think it is related to some of this adenoviral work. It is a chemical experiment where one dose of vinyl carbamate was given at one time in life, which was very early, and was given at two doses, and there was no further treatment and the animals were held and then they were periodically sacrificed over a period of time up to 30 months.

(Slide.)

This is a generic depiction of foci of cellular alteration and they are expressed in terms of multiplicity. We have standard sections that we take that represent a certain amount of real estate and this is simply a count of how many foci were present in that standard amount of real estate. For the higher dose there is a rise up to close to 15 or 16 foci and then a decline and then in the lower doses there is a rise and a decline that occurs later temporally and then there is a very modest response

in the controls.

(Slide.)

This is the prevalence of hepatocellular adenomas in the same experiment. The high dose shows adenomas occurring early. This is a reasonably heroic treatment even though the animals only got one dose early in life and so there is 100 percent prevalence. Carcinomas follow behind with a high dose showing the occurrence of carcinomas before the low dose and certainly well before they control.

So the reason for showing you these generic sort of pictures is to look at one composite which is, for brevity sake, made up of three strains, the B6C3F1, the C3H, those are two susceptible strains, and the C57 Black-6, which is more resistant.

(Slide.)

And here they are. The B6C3F1, the C3H and the C57 Black-6. And you can look down and across and for the two susceptible strains there is a reasonably robust focus response and a very prominent hepatocellular adenoma and hepatocellular carcinoma response. In the C57 Black the response is much more modest and please note that it occurs later in time. There is a latency issue here that is very important. So besides does latency is important.

(Slide.)

In terms of multiplicity of tumors in the liver, if you look at the blue bars this is the control B6C3F1, the low dose has more tumors per liver and this is per whole liver, six, and the higher dose has up to about ten. For the other two strains depicted here, as the dose goes up, the multiplicity of the response goes up and this is multiplicity of tumors.

(Slide.)

So let me just summarize where this is. All strains examined -- and we have examined more than the three I have shown you -- are susceptible to the development of hepatocellular neoplasms and they all go through this progression. I do not know of any exceptions.

(Slide.)

There are strain differences that are present in terms of the sensitivity and these strain differences exhibit a reduction or not in latency. Certainly a reduction in the latency versus a control but there is a variability between strains in terms of how quickly the tumors develop. And treated animals always seem to have more tumors per liver than the controls.

(Slide.)

So there are some general conclusions to take away. All strains can develop liver neoplasia. As I said before, it is reasonably common. All strains can respond to hepatic carcinogens. I do not know of any exceptions. If you wait long enough you are going to see the response and it will be pretty clear. Strain differences are primarily differences in latency. The dose if it is a carcinogen you are using influences latency and the multiplicity and sex differences, and I did not mention this. Males get more of a response than females. Sex differences are largely differences in latency also. The males will get neoplasms earlier.

(Slide.)

There is one additional conclusion that I think might be germane here. If you terminate your study early you probably are not going to detect a response in a strain that is not very responsive. So there are a lot of chemical bioassays that are done in C57 Black mice and they are terminated at 18 months because at least some regulatory authorities permit 18 month duration. At which point in time you usually

do not get liver tumor response in the C57 Black. And the sponsors of those studies like that. We do all our studies for two years and I assure you that if the studies were longer than 18 months in C57 Black there would be a few more responders than what show up.

(Slide.)

I cannot say much about hemangiomas and hemangiosarcomas because I think I restricted my comments mostly to those that occur in the liver but I will make the point that they are considered systemic neoplasms. They are not considered organ specific. They are related to the vascular system and the endothelial lining cells, which can occur anywhere in the body. So we lump them all together. If we get subcutaneous and uterine and splenic and hepatic and even brain hemangiosarcomas, we look at those in totality rather than partitioned out by specific organ.

In the liver and in general in the body they are low incidence tumors particularly in controls. There are some hepatic carcinogens that actually produce them in the liver.

(Slide.)

Hemangioma in the liver is characterized by this low power photomicrograph. Lots of spaces and there is a thrombus here that are separating hepatocytes that are becoming atrophic and each of these linear arrays of atrophic hepatocytes are lined by endothelial cells.

(Slide.)

The malignant tumors are more complex in their structure. The hemangiosarcomas with a great deal of destruction of normal hepatic parenchyma. In other words you do not see the atrophic hepatocytes. You can find solid areas proliferating plump endothelial cells and a lot of degeneration of the adjacent hepatic parenchyma.

(Slide.)

With respect to genetically engineered animals, and they are mostly transgenic, and the development of hepatocellular neoplasia, in general I can say it is quite similar qualitatively to what we see in conventional animals.

(Slide.)

There is a whole shopping list of possibilities here. I am going to just summarize, and I am sure I missed some, too, what the primary distinction is in the genetically engineered mouse hepatic neoplasia versus the conventional.

I said they are qualitatively similar. They typically occur within a shorter period of time depending on which animal it is. There is an increased multiplicity compared to conventional animals and there is often, almost exclusively I almost want to say, marked dysplastic change in the hepatocytes that accompanies this process, which makes it different from what occurs in conventional animals.

We do not know too much about hepatocellular neoplasia and genetically engineered animals. Depending on what you are going to find in all of the studies that you are going to be dealing with and presumably doing in the future, and because part of the information I got before I came here used the word "hepatoma" which I have not heard in so long, I thought that maybe some people need to just be made aware that there are some classic references.

(Slide.)

I do have these listed out if somebody wants to get a copy of the citation so you can find these. We abandoned the word "hepatoma" in the '70s and I was surprised to see it again.

Thank you for your attention.

(Applause.)

DR. JOHNSON: Questions for Bob?

DR. CRAWFORD: A quick question. Could you -- although the answer may be wrong, could you comment on molecular markers of premalignant change because histology is the gold standard but clearly there may be things that you can detect earlier? There may be.

DR. MARONPOT: In mice, yes.

DR. CRAWFORD: In mice.

DR. MARONPOT: Yes. Unlike rats. In mice we struggled with this for a long time and the glucose-6-phosphatase deficiency was the primary marker that was touted to allow you to identify a focus before you might be able to see it otherwise.

First of all, that is a difficult thing to do. You have to have frozen sections. Not everybody freezes tissues suitably for making sections. And our experience has been the H&E is, in fact, just as reliable, if not more reliable, the morphology is good, and we do not have any trouble finding H&E foci. So that is our preferred marker so to speak even though it has been around for 100 years.

DR. KAY: I am going to get back to this question about these glucose-6-phosphatase deficient individuals. I mean, almost 100 percent of those have hepatic adenomas and many of them go on to get HCC. It is interesting -- although there is no data -- that people argue that the mechanism may be due to insulin -- over insulin secretion. It is a storage disease although it is different than this particular lysosomal storage disease. So I guess is there any way to bring this mechanistically together? Could this have something to do with the IGF II receptor or is this -- and the fact that there is a lot of storage material in cells? Or do you think this is probably totally unrelated?

DR. MARONPOT: That is a difficult thing to answer because you just do not know sometimes if it is just spontaneous errors in DNA polymerase when the slow turnover that is going on is occurring and that is generally what is -- unless you have got a raging carcinogen that you have been testing and that is generally what people invoke.

I would never rule out Randy's mechanism as a possibility but I cannot explain that. I do not know what the good answer is for that question. It is a good question.

DR. JOHNSON: Phil?

DR. NOGUCHI: A mundane question but -- it is unfair because you only saw one histology slide but will you say that the histology that Dr. Sands showed would be typical of this dysplastic hepatocarcinoma that is seen in the genetically altered animals because it certainly did not look like any of the hepatomas -- I would call them hepatomas. I am sorry. But hepatocellular carcinomas that you showed which still retain a certain amount of architecture and regularity.

DR. MARONPOT: Yes. That was over exposed Kodachrome and that was difficult to see. I strained and I thought that there was clear morphism in the nuclear size but that was a difficult one for me to pick out. And it was a high power so you do not really get to see much except a very narrow field. So I have to come away having some question and, like most pathologists, I would say let me see the glass.

DR. JOHNSON: Mark, do you want to say anything about that?

DR. SANDS: Well, I am not a pathologist but several of the low powered sections that you had up there look strikingly similar to what we see on low magnification and again I am not a pathologist so I am not going to make any interpretations but I would be ecstatic if you would be willing to look at these slides because, you know, Carol -- Carol is a very good pathologist. She has looked at these mice for 12 years but she is not a mouse pathologist per se who specializes in tumors. I would be ecstatic if you would look at them.

DR. MARONPOT: As a mouse hepatologist I would be happy to do that.

(Laughter.)

DR. JOHNSON: A collaboration born. One more question.

DR. _____: Yes. Presumably in all these studies that you have done you have used a number of different components, genotoxic, nongenotoxic, and one assumes there is a lot of different vehicles that you have injected. What is the data in terms of the shifts in terms of just instrumentation injection of various, say DSMO or glycerol, other sort of vehicles actually has an impact on these tumors?

DR. MARONPOT: That is a good question. I am not sure I can answer it completely so I will do the best I can. Most of our studies are dosed feed so it is in the diet and certainly diet is a factor but our diet has been pretty consistent. Gavage studies were initially predominantly corn oil and that caused some other problems nutritionally actually so we have gotten away from that. And that leaves the inhalation studies, which is an entirely different route. Those are the only -- well, occasionally a few animals -- animal studies were done with drinking water administration.

All of them produce hepatocellular neoplasms to about the same degree and, of course, it depends on the carcinogen and the control animals also in the air exposed or the normal diet have the normal component. We do run database historic control data separated out by route of administration and for liver neoplasms, primary liver neoplasms, there is not a lot of difference. There certainly is for other tissues though.

DR. JOHNSON: Well, we are going to break for lunch now. We still have lots of questions remaining on the agenda.

Lunch is downstairs. You were provided a ticket, I believe. You can either eat a buffet and I believe you can order off the menu. The only constant is that we have to be back at 1:15. Thanks.

(Whereupon, at 12:34 p.m., a luncheon break was taken.)

* * * * *

A F T E R N O O N S E S S I O N

ROUNDTABLE DISCUSSION (Continued)

DR. JOHNSON: Well, we are ready to resume the roundtable discussion and we are on to question number two that has five subparts to it.

(Slide.)

"Please discuss the factors that could account for the observed tumors in the gene transfer study in the MPS VII mouse model, including (a) the AAV vector; (b) underlying MPS VII disease coupled with increased longevity of the affected mouse; (c) over expression of GUSB and saturation of the M-6-P receptor; (d) mouse strain; and (e) age of mouse at time of transduction."

So those are the five points that we have got to discuss and maybe there will be others added to that as we progress through that. So I am going to open it up to discussion on the AAV vector as the positive agent in this model. Now we covered some of that, I think, in question one.

So does anybody have any comments regarding -- any further comments, I guess, regarding the role of the AAV vector? I do believe we have covered a lot of that in question one.

DR. FLOTTE: I have just one point that I did not remember to make when I gave the talk which was that one of the studies that we pulled together there was a study that Nick and Al Lewen performed attempting to use the vector to generate tumors. Did you want to talk about that, Nick?

DR. MUZYCZKA: We did two experiments. One was to make ribosomes to RB3 and 21, and then make vector and inject those into newborn mice in an attempt to essentially kill tumor suppressor genes and thereby create tumors. And the second experiment we did -- in the second experiment we did a similar strategy but this time we actually made a library of ribosomes with a complexity of about 50,000 ribosomes in the hope that we would get combinations of tumor suppressor genes and create tumors.

And the third experiment -- and that is -- we did some 14 newborn mice that way at very high systemic injections of vector and in the case of the RB and the p53 ribosomes we had evidence from in vitro studies that those were effective and we were not able to find any tumors. They wound up being part of the control animals that you saw that Terry Flotte presented. No tumors of any kind.

The last experiment we did was in a very limited number of five -- four actually -- p53 knockout mice. Again the idea here was could we go in with the library in the p53 knockout background and create a tumor. And we did, in fact, get some tumors but the -- when we examined the tumors they were first of all, approximately -- showing up at approximately the time that you would expect. They were lymphomas expected in normal p53 background. And the vectors had a GFP gene in them as well as the ribosome library and the tumors were not positive for GFP so they did not appear to be due to factor expression.

So we tried to make a tumor and we could not.

DR. SAMULSKI: To move things along, I think what is probably appropriate is with all the data shown today, all the vectors that used the TR there is no other examples of insertional mutagenesis with any AAV vectors, either in dogs, monkeys or other rodents. The closest study that comes to Mark's analysis is the one that Terry did where he has the same vector and the same promoter with a different transgene and those are zero for 22, although the numbers are small. And so it looks like we are getting down to the transgene in the model as the next thing that needs to

be evaluated as well as whether these numbers are sufficient to be able to conclude that that TR promoter cassette is not a player.

DR. CRAWFORD: If I could put some additional data on the table to support that. The reason I asked the question earlier about molecular markers is if you are going to go the extra mile and see if there is any way you can detect premalignant change, it might be worth trying to. And I should note that the human literature in terms of detecting premalignant change for hepatocellular carcinoma is littered with failed tests. That being said, we ran on these mice, which were the neonatal injected 13 months out, we ran ploidy analysis as well as PCNA, proliferating cell nuclear antigen, and had absolutely zero indication of, if you will, premalignant foci that had not yet become evident by H&E staining.

DR. JOHNSON: Mark?

DR. KAY: Getting into this issue of the model, I mean I was intrigued by Bill Sly's data that there was a transgenic line that actually made more enzyme and they did not get tumors and thus there was a discussion about insertional mutagenesis in that particular mouse where there is an insertion into every single cell.

But my question is related to the issue could you make so much enzyme that you are getting more crystals? I mean, between those two lines do you get more crystallization such that these molecules would not be available for uptake by this IGF II receptor and, therefore, be less susceptible if we accept the receptor as a possible model.

DR. SLY: I think it is important to know that there are two mice, two lines. One that -- both which have transgenes which are inserted. And one of which leads to tumors and one of which does not. So enormous levels of over expression of the enzyme itself does not lead to tumors because the line which expresses the highest does not get them. It is the other line which expresses slightly lower that does get them.

DR. KAY: Do those animals get more --

DR. SLY: Sorry.

DR. KAY: Do those animals have more crystallization in those tissues that over express, the ones that do not get tumors, because they are making more?

DR. SLY: I think the pathologist -- the same pathologist, Carol Vogler, she could not distinguish histopathologically in most tissues between the tumors and the -- I mean, between the tissues and the two -- in most tissues. We did most -- actually we spent most of our time analyzing the highest level producer.

DR. KAY: Is there anything in the serum?

DR. SLY: There is 2,000 times normal levels in serum in both animals.

DR. KAY: Do you know what the copy number is?

DR. SLY: About four.

DR. KAY: Four.

DR. _____: Mark, on that same issue, the fact that there is soluble enzyme in the serum would suggest that there is enzyme available to bind the Man-6 phosphate receptor in addition to the stuff that is crystallized.

DR. JOHNSON: In the back?

DR. DAWES: Yes. One thing I have noticed is that there is no discussion of the AAV capsid as a possible contributor and there actually is sort of a crazy concept I have been developing. There has been a recent report at a parvo virus meeting which has not been published yet by Zadori, et al., that parvo virus capsids

may have phospholipase A2 activity.

Now with the MPS VII there is a high amount of heparin sulfate so it is possible that you are bringing in more capsid in these cells than you would for other disease models. And there might be some sort of overdose of PLA2, which is known to be involved in cell proliferation cascades and things like that.

DR. JOHNSON: That is an interesting thought. I certainly had not come up with that thought so we need to write that one down.

Jude?

DR. SAMULSKI: I should just add there is now reagents that could test that, Roland, if you wanted to pursue it. There are empty capsids carrying out genetic information that could be tested.

DR. JOHNSON: Phyllis?

DR. GARDNER: If I go back to the final talk, I am not sure how to pronounce your name, Dr. Maronpot, it seemed to me that this transgene with this construct, with this method of delivery, you would define that particular administration as carcinogenic. That looked classically carcinogenic. We do not know what factor in there is the carcinogen.

So then I was going back to every other administration and Terry Flotte -- I guess I heard that Terry has the 22 mice with the same construct, different transgene. What strain -- and the other thing I heard from Dr. Maronpot was that it is strain specific in terms of latency, et cetera.

So how long were the -- how long were Terry Flotte's mice followed and what strain were they, and what sex? I think all those things matter in terms of --

DR. JOHNSON: Terry seems to have disappeared.

DR. GARDNER: That is bad.

DR. MARONPOT: Maybe I can make a comment, too, apropos to some of those comments. I might take it a step further. This is a little naive maybe but it would seem to me if we had some concern about the safety of the vector, just the vector, why not test it in a sufficiently sensitive strain that -- because you suspect a liver problem for a sufficiently long period of time and make sure they do not have helicobacter or something else that is going to synergize or confound the interpretation? It seems to me that that might be a first step if somebody would be willing to do that.

DR. JOHNSON: Terry, Phyllis was asking about the strain of mice that you are using and length of time they were kept.

DR. FLOTTE: Yes. The compiled data in that slide, which I think Kelly was going to pull off the computer and print out for people, included several different strains of mice. Most of our neonatal injected mice were Black and Tan BTBR strain. We also had some of the -- I believe it was some Black-6 mice that were injected as neonates but I will have to double check that. Then we had a large number of C57 Black-6. We also had some BALB-C. We also had some of the Pompei's disease from Barry Byrne's work, which is the GAA knockout mice that I believe are on the 129 background. So it is probably four or five different strains at least and that was in the table.

As far as how long they were followed, in the summary table we pointed out that we started with -- we had a total of 137 mice. We had, I believe it was, 51 mice that were past -- that were 11 months or longer and then 39 mice that were at 13 months or longer. And within those there were ones at and beyond 18 months. And then that broke down the number that Jude focused in on. In terms of

the 22, those were the newborns that went out past 13 months.

But obviously if they were injected, as many of them were, at three weeks and then followed for -- out to 18 months or longer, their duration of exposure was comparable and so that was why we were looking at the 51 or the 39. The duration of exposure -- we broke them out as well in case someone was interested in the issue about whether the neonate at the timing of vector exposure would have been a relevant issue, not just that it was three weeks longer of vector exposure in terms of developmental issues.

DR. SAMULSKI: Terry, can I just get you to add to that some information? If this was a toxicity type situation related to vector dose, were your animals dosed at a higher or lower dose than what Mark Sands' animals were?

DR. FLOTTE: There was a -- the newborn IV injected animals --

DR. SAMULSKI: The newborns.

DR. FLOTTE: -- were at about 50-fold higher dose.

DR. SAMULSKI: So you had a 50-fold higher dose.

DR. FLOTTE: Let me rephrase that. The higher dose group -- the newborns were about half of that dose and then about half maybe at a tenfold higher dose.

DR. SAMULSKI: But everything was higher.

DR. FLOTTE: Several of the cells in the table included different dose levels. Obviously we are looking for dose related biological efficacy as well as dose related toxicity.

DR. SAMULSKI: So, you know, if you try to tie some of the toxicology data with compounds with what you have done without intentionally trying to test this premise, the dose that Mark used where he saw tumors was a lot lower than the dose you used in which you have not observed any kind of tumors.

DR. FLOTTE: Right. So if it is important -- I mean, obviously it is an important issue for any toxicologic effect that you would -- most of them you would predict would be dose related and this was not.

DR. SAMULSKI: Right. So I think it focuses back --

DR. GARDNER: It seems to me -- I do not mean to interrupt but it seems to me that it behooves one to do the experiment now prospectively to look at it strain specific, dose specific and latency specific before you finalize it because it is just too mixed up, all the day, even though it sounds reassuring.

DR. FLOTTE: Well, you know, I just want to point out -- I mean, you know -- I thank Phyllis for bringing that up. I mean, you know, we had a series of meetings a couple of years ago where we were trying to design just such a prospective study and, hopefully, Anne Pilaro will have a chance to comment on what some of the elements of the design of that study would be.

At the time a couple of years ago it was felt that it was -- we would look selectively trying to pay attention to integration, trying to keep all our data in a way that could be pooled, and the NGBL has taken a lead in creating a mechanism to pool the data so that when situations like this came up, we would be able to sit around the table and compare data in a meaningful way.

Maybe the time has come to -- you know, which we thought might have been premature a couple of years ago, the time has come now to do a large prospective study. Obviously it is the only way we will really have a definitive conclusion.

DR. JOHNSON: Phyllis, does that answer your question?

DR. GARDNER: Yes.
DR. JOHNSON: Any other points on this particular issue? Okay. Let's move to number -- oh, sorry.
DR. SLY: I just think in view of the large strain differences in sensitivity you cannot make an N of 43 when you have six strains. It is an N of -- it is six N's of about five or six animals.
DR. JOHNSON: Well, put. That is exactly right.
DR. MARKERT: If you are discussing question number two, are there any other issues, I think the issue that came up --
DR. JOHNSON: Actually we are on number (a), AAV vector, under that is where we are.
DR. MARKERT: Okay. I have a new one I will add later.
DR. JOHNSON: Okay.
DR. FLOTTE: Could I just respond to Dr. Sly's point? It will be important for people to see the data because that is not really the way it plays out. I mean, these were individual experiments done in different ways so actually when you look at the long-term animals, there are clusters within individual experiments that were intended to go long-term.
So, for instance, out of the 22 long-term neonatal IV animals that we have, I think 18 of them were from one particular Black and Tan newborn IV study with the CB promoter alpha 1AT vector done at 50 times higher dose. So actually they are not randomly distributed between the groups so a lot of the dropout in our dataset going from the total number of liver exposed animals to the very long-term were where entire studies were terminated by the PI to look at other endpoints.
DR. JOHNSON: Okay. Let's move to the second point, which is "Underlying MPS VII disease coupled with increased longevity of the affected mouse." So in other words is the disease itself coupled with making these mice live longer responsible for the observed tumors?
Mark, why don't you tackle that one?
DR. KAY: I think it is hard to argue that with the protein therapy and the bone marrow therapy that making the animals live longer makes them prone for these tumors because you have two completely different routes of therapy and there does not seem to be this tumor incidence unless the animal model people want to add some specifics.
DR. SANDS: I agree. I mean, again we do not know -- obviously we do not understand the mechanism but this is one thing that made all the bells and whistles go off when we first discovered these tumors, was that we have been studying this -- it is not like we made this model a year or two ago. We have been studying this model for well over 12 years with lots of different therapies, lots of different approaches, and we have never seen anything like this.
So I think the simple explanation that they just live longer is not one of my favorite hypotheses, although again the comparisons are apples and oranges. Different routes of administration, different cells have enzyme in it, different levels at different times. There is not a perfect comparison. The perfect comparison would be to let an MPS VII mouse live for two years. They do not live that long. So short of that I am not sure what -- how to get at that answer.
DR. JOHNSON: Any comments?
DR. KAY: I mean, I agree. I mean, if I look at all this data together -- I mean, to me, it seems like it has something to do with this specific over production

of a large amount of enzyme in a very small number of hepatocytes. I mean, obviously with -- if you look at all the different experiments they are not all well controlled because they were done at different times in different labs. But essentially if for a minute we assume that Terry's data controlled for the promoter enhancer -- he also injected huge amounts in neonatal mice, saw no tumors -- there has been a plethora of data where people have injected large amounts of AAV in the liver and not seen tumors. There is this transgenic mouse issue and the bone marrow issue, and you do not see tumors.

If you look at the one variable that seems to be suspect, it is the issue of possibly expressing a lot of protein from a very small percentage of cells. And at the time when we were thinking about all this, I would not have any reasonable way to explain it but when I hear Randy's data it makes sense. Whether or not it is true or not, I just -- it needs to be tested.

DR. JOHNSON: Yes. I think it is also worthwhile to think about another aspect and that is that this is a foreign transgene. This is not the mouse GUS gene. We know, I think, from Bill Sly's data that they are probably making immune response.

Do we know whether these mice are making immune response?

DR. SANDS: In this particular experiment we did not analyze the immune response in this particular experiment but in previous experiments with recombinant enzyme therapy and in our first report using intravenous AAV administration we looked for antibodies. We have never seen antibodies in this animal if you initiate the therapy at birth.

Now, as Bill pointed out, if you initiate the therapy in a young adult or an older animal, you do get antibodies made but everything we have ever done in newborn animals we have never detected any antibody response.

DR. KAY: I just want to add a caveat. Something we were discussing earlier is that the immune response to protein delivery versus protein being made in a cell is going to be different and if you are using bone marrow or transgenic mice the immune response is probably tolerized. So I think that, you know, the cells that are actually making the protein and how much is being made and where it is being made is going to influence this and nobody knows in this situation.

DR. SANDS: But in our first AAV study we did not see any antibodies and it is basically the same. I mean, the results are really very similar. Similar -- it looked like similar levels of expression in each hepatocyte and that sort of thing. We saw no antibody response.

DR. KAY: But there is other immune responses besides antibodies that if they are -- if it is responsible for tumors at some level, it may not be antibodies. There are cell mediated immune responses. There are lots of other types. So just to keep it in mind.

DR. JOHNSON: Yes. I think that some of the inflammation that you are seeing could well be lymphocytes that are directed towards epitopes that are not native to the mouse and I guess I am a little surprised that in your first study you did not find antibodies to beta glucuronidase and you said you did not because in every -- in almost -- in every single experiment that we have ever done with AAV that we have put in, we have not done neonatal mice, the caveat, but in any other species that we have ever put it in and the transgene is foreign, we get a tremendous immune response to the transgene.

DR. SANDS: Sure. And we were actually surprised when we did not

see an antibody response especially to the enzyme replacement studies because we get huge boluses of enzyme and, you know, they are -- and we give it continually so basically we just keep priming them. And if we start at birth we do not see it. We never have.

DR. SLY: There is an important caveat there. All those enzyme replacement studies were done with the mouse enzyme.

DR. JOHNSON: That is important, yes.

DR. SANDS: It is important but the mouse also probably does not express any protein. It has virtually no RNA so it does not take much to create.

DR. CRAWFORD: Mark, when you talk about the expression of the enzyme as being uniform across the liver, that is uniform in its sporadic nature, is that correct?

DR. SANDS: Yes, that is correct. It is not in every cell. It is in isolated cells uniformly around the liver if that makes sense.

DR. CRAWFORD: And the next question is have you run immunohistochemistry or in situ on the tumor tissue?

DR. SANDS: We have done our histochemical stain on one of the tumor samples and the data are hard to interpret. The endothelial looking cells are very -- have a lot of enzyme activity associated with them, which could be just uptake from the serum. There is an awful lot of enzyme activity in the serum.

The hepatocytes stain faintly for the enzyme. Again my interpretation of that is that those hepatocytes are taking that up from the serum also because when see a cell that we think is transduced it is just producing an enormous amount of enzyme activity.

DR. JOHNSON: You know in situations where there is chronic inflammation in the liver you could create islands of inflammation that would lead to conditions for developing hepatocellular carcinoma. So I think if you have got an immune response that it is worthwhile thinking about. So one of my questions would be would it be worthwhile to do this using the GUS gene, the mouse GUS gene rather than the human GUS gene and see if, in fact, there is a difference in the outcome of the experiment with the caveat -- you know, all the caveats aside about how difficult they are to breed and the money. Money is no object here, right? So I think that is worth exploring. I mean, it is a testable hypothesis that could be really looked at very, very cleanly, I believe, and I think that is something worth looking at.

Jude?

DR. SAMULSKI: Can I make a different suggestion? I think Bill has provided the excellent model. The one that has the human gene in there as a mutated form that clearly does not generate antibody would allow Mark or someone to go in with the exact same vector that has caused tumors and very exquisitely determine if the immunological component is what is responsible. And clearly we want to reproduce the data in the original animals but this animal would take out that whole concern without changing any vector transgenes or anything. I think that is a recommendation as far as if we want to try to resolve this experimentally.

DR. SANDS: I think the only caveat there is, as Bill showed in his slides, these mice now have a beta glucuronidase storage deficiency and I am not sure how that would affect.

DR. SLY: Sorry, Mark. It is the first transgene which makes a tiny -- a relatively small amount of that enzyme. It has no -- it has MPS VII but it does not have the over production disease. So that is why we made that mouse to be a

candidate for trying things that would be used in human trials.

DR. KAY: I am still intrigued by this receptor issue and I wondered if there are any other lysosomal storage disease animal models in which the enzyme is normally taken up by this receptor and whether as an independent experiment somebody could over express that in AAV and whether that would be useful or not.

DR. JOHNSON: I want to point out that we are moving to question (c) with that segue.

DR. _____: I think I can answer Mark's question. So as Terry alluded to, some of the animals in the UF experience were a glycogen storage disease Type 2 mice. They are deficient in acid alpha glucosidase. They have not been observed to develop any tumors. It is not part of the natural history of a disease. And in mice either transgenics on the knockout background or vector treated mice, we do not observe this phenomenon.

DR. _____: (Not at microphone.) (Inaudible).

DR. _____: Yes.

DR. SLY: What is the N? And the period of observation?

DR. _____: Well, the transgenic mice are probably a year old by now. The vector derived mice have been out past this 13 month time point.

DR. KAY: So this is AAV?

DR. _____: Yes.

DR. KAY: How many mice, Barry?

DR. _____: There have been about two dozen mice in the vector experiments, both adults -- and the ones Terry referred to are neonates. And in the transgenics there is a colony of about 30 mice. Both express from liver and from muscle. So we have two regulatable transgenes either specific to liver or muscle. So they over express acid alpha glucosidase at about 200 times the background level.

DR. SLY: I am a little confused here. Could you clarify what you mean? You are doing this on a knockout background?

DR. _____: Right.

DR. SLY: And you --

DR. _____: So they are GAA- minus mice, which have been mated with transgene -- with transgenic mice also on the knockout background and they are expressing either a -- they are through the TEC control system expressing either a liver specific or muscle specific transactivator. And the third line has the TRE control acid alpha glucosidase.

DR. SLY: Okay. But I think those are interesting for other reasons but I do not think they are relevant to this.

DR. _____: No. The question was whether over expression of a lysosomal enzyme, which is targeted to Man-6 phosphate receptor, is related to a problem. So these mice have serum levels of acid alpha glucosidase, which is not normally observed in the serum.

DR. SLY: How high are they?

DR. _____: What is that?

DR. SLY: How high?

DR. _____: The serum levels -- you know, we cannot relate them as percent of normal. They are sufficient to produce in the tissues a 200-fold over expression of the enzyme.

DR. _____: That is pretty impressive.

DR. JOHNSON: Over here?

DR. GORDON: Yes. Just a thought that might be useful to point out.
DR. JOHNSON: State your name and affiliation.
DR. GORDON: Dr. Gordon from RAC. I am a RAC member.
There are a couple of transgenic models where proteins have been expressed at extremely high quantities from hepatocytes using like albumin promoter enhancer complex where because they are germ line integrations immunology is not likely to be a factor and these mice get hepatocellular carcinoma. One case is the hepatitis B surface antigen which is billed as a model for hepatitis B induced hepatocellular carcinoma even though the issues are slightly different because the animal does not have the same florid immune response that a human does that is infected with hepatitis.
The other is alpha 1 antitrypsin deficiency where there is a mutation in the alpha 1 antitrypsin gene which inhibits its release from the hepatocyte, although the genes still function, those animals also get hepatocellular carcinoma. There is not an immunologic mechanism there so it is not entirely clear what is going on. When you make a lot of protein from inside of a hepatocyte, in that particular paradigm it is associated on at least two other occasions with hepatocellular carcinomas.
DR. KAY: But in that allele of alpha 1 trypsin deficiency those livers are undergoing some chronic regenerative changes because the accumulation of that protein is a cell lethal event similar to what happens in tyrosinemia type 1 where those animals all develop HCC and so do the patients.
DR. GORDON: Well, it is an irritant. I am not so sure that the causative mechanism is that it actually kills the cells because in the hepatitis B surface antigen mice I am not sure how many are killed but it is also true that the prodrome of both of those strains is inflammatory changes in the liver, reactive changes, alpha fetal protein production, new mitotic activity in the liver, even cells that do not seem to ultimately contribute to the foci of carcinoma. I think actually in these mice of your's AFP studies would possibly be informative.
DR. JOHNSON: Jim?
DR. CRAWFORD: Assuming that we have actually segued now to the third part, I would like to put a hypothesis on the table and ask for Randy's comment.
To the extent that there is high local and sporadic production of the transgene, we also know that adjacent hepatocytes can take up the enzyme, and it is conceivable that a high local producer, a high local hepatocyte producer could, in fact, induce a second hit on neighboring hepatocytes which are not carrying the transgene. To the extent that both the producer and adjacent hepatocytes undergo clonal expansion you could explain the low but positive virus copy number. In essence, having a field effect where an apparently single hepatocellular carcinoma is, in fact, arising from a field effect in a local area. One cell of which is positive, other cells of which are negative for the transgene.
DR. JIRTLE: Yes, I mean all this is possible. Because if you are occupying the receptor by something other than what it is normally seeing high levels of, you are going to affect the ability to degrade IGF II, for example, or activate TGF beta, or as I said before, even if that cell is mutated or such that it is being targeted for being eliminated by cytotoxic T cells, it might not take up granzyme B as readily and, therefore, you evade the immune response.
These are all hypothetical but they are real. I mean, you do not need actually a mutation in the gene if you swamp that receptor locally.
DR. CRAWFORD: But it is a potential explanation for the data we

have seen today. It is a bit far fetched.

DR. KAY: You still have the problem potentially with the over producing cell lines, I think. Why don't they hit that one line? Are those in the same genetic background?

DR. SLY: Yes. They were actually made on an FVB transgenic background and then bred ten generations on B6.

DR. CRAWFORD: Both of them?

DR. SLY: Yes, right.

DR. KAY: But remember the --

DR. SLY: Actually I should say the tumors developed earlier on before they were completely on B6 background.

DR. KAY: But remember the point that the liver cells -- the hepatocytes are only making seven-fold higher levels.

DR. SLY: No, I think I agreed with you but I was wrong. they are making 700-fold. They are not making 8,000-fold but they are making 700-fold.

DR. KAY: Okay. Because I thought the slide said seven.

DR. SLY: Did it say seven-fold?

DR. KAY: It said seven.

DR. SLY: Oh, I am sorry. Okay. All right.

DR. KAY: So the point is with AAV, like for example with hemophilia, you know, we have data that the amount of Factor IX may -- you can do some rough calculations -- is about 50-fold higher than what it would normally make or above that.

So I would suggest that possibly n these AAV treated mice that the Amount being made per transduced cell is probably much higher than seven-fold.

DR. SAMULSKI: You know, the model is attractive but I think it is going in a direction that we cannot answer. One is the protein therapy where they are putting in enormous amounts of protein should mimic the exact same thing as a neighboring cell pumping out a lot of product.

DR. SANDS: No. No, because you are getting these huge peaks and valleys. What we have shown is when you do an intravenous injection the enzyme is cleared very, very rapidly. So, in fact, you probably do swamp out the receptors very quickly but then it is gone. And the situation that we are describing here is a continual exposure of the receptor.

DR. SAMULSKI: I like that model.

DR. CRAWFORD: Order of magnitude also becomes an issue here because in quantitative work that we have done on the alpha 1 antitrypsin model it is less than five percent and sometimes only one to two percent of hepatocytes that are expressing, number one. So that is a twofold order of magnitude -- sorry, 100-fold change right there.

And then the next is how much is an individual cell expressing? And we do not have that data but you can go up orders of magnitude fairly quickly.

DR. KAY: The real time PCR data would suggest that the number of transduced hepatocytes is very low and the other point that you had brought up about the expressed cell kind of coming along or also proliferating, I think is less likely because two of the four tumors do not have any AAV.

DR. CRAWFORD: Right, which means -- I mean, if we were to play this out -- in two cases the expressed cell also went clonal and in the others it did not.

DR. KAY: Except it is still one-tenth or less, one -- you know, fiftieth.

Yes.

DR. CRAWFORD: Right.

DR. JIRTLE: I was just going to make one more addition that -- I do not know if it helps or hurts but we also looked at tyrosinemia patients that developed hepatocellular carcinomas. They are relatively rare and I think we have been able to get 12 tumors that we were able to look at and every one of them had the IGF II receptor mutated, 100 percent.

DR. JOHNSON: Also, let's suppose that it turns out to be this receptor mediated phenomenon, make the point again, Randy, about the difference between a human and a mouse.

DR. JIRTLE: Yes. I made that in my talk but I guess i can do it again. If this ends up being the mechanism it is going to be much harder to swamp this out in humans than in mice because you have two copies that are totally functional.

So this might be -- that is why I started -- that is why I started off this thing -- my talk with a story, which ends up being sort of like a joke, you know. Is there a problem with mouse liver tumors? It might be that this is actually just a problem with mouse liver tumors even if we knew what it was because we are really very different from the standpoint of this locus. We have two copies that are functional.

DR. BREAKEFIELD: That does not seem to me -- just in terms of numbers, when you bring a vector in you are expressing so much of this enzyme and now you are talking about just a twofold order of difference but you are talking about making 50 times as much of the enzyme. I do not see how that twofold difference would make a big difference.

DR. JIRTLE: Yes, but apparently a loss of a single allele just even in carcinogenesis is a lot --

DR. BREAKEFIELD: But that is at low levels. We are talking now about elevating them up very high so then you are swamping out, right?

DR. JIRTLE: Yes, but what I am saying is that when the liver tumor in that environment of chronic cirrhosis there is a lot of inhibitory factors that are being produced there also. And these cells do get a growth advantage just by losing a single allele. So my point is with this receptor -- you know, it is like a quarter inch on the end of your nose that really is very important. I think it is maybe the same thing here. Losing one allele is very, very important. Much more so than what you would think.

DR. JOHNSON: In the back?

DR. _____: Yes. At the risk of seeming very naive in the presence of this very sophisticated theoretical discussion, I remember being taught in medical school that when you get an unexpected laboratory result the first thing that you do is to repeat the test. And it seems to me that what it behooves the entire field is to have another lot of the exact same virus made, the exact same AAV vector, and put into another group of the exact same strain of mice, and see if the results are repeated.

Because as far as I can see -- I mean, from what I have heard it is not clear to me that this product was made up to GMP manufacturing standards, which means that it was probably not exhaustively tested for adventitious agents. I mean, exhaustively tested. And it could be something as simple as that that is causing this phenomenon.

This discussion, while very illuminating, may be irrelevant to AAV.

DR. MUZYCZKA: Yes. Let me agree with you. Okay.

(Laughter.)

DR. MUZYCZKA: On the first point I also when I look at the numbers here think that we need to replicate this experiment and your point is extremely well taken.

There could be a variety of reasons. We mentioned helicobacter in an animal colony and you mentioned a potential contaminant in the particular preparation and virus, and all those are very reasonable. We may never see this effect again if we repeat the experiment.

On the question of the virus preparation's particular stock, you are absolutely correct. This was not -- this was -- I guess you can call it a GLP prep. It is not a GMP prep. We did not exhaustively test it. It was a preclinical prep for not even a tox study at the time. So there was no reason to do extensive testing on it.

DR. FLOTTE: Can I just reinforce the last point that Nick made is that this -- I mean, obviously this study was not designed to determine whether there is a potential carcinogenesis risk from recombinant AAV. I mean that is a question most of us have been interested in but most of us have been engrossed in trying to test the feasibility of this system to cure diseases in animal models as Mark successfully did in the first part of his work.

So the study was not designed for this and I think many of us feel very strongly that it needs to be repeated prospectively with all the appropriate controls considering some of the discussion that goes on here and then perhaps other long-term carcinogenesis studies that are more -- that are designed to be more generalizable certainly also need to be done. Although I would -- I personally think this discussion today is very useful because this study will take two years after injection of the virus.

We have clinical studies going on currently and, you know, it will obviously take us a while before the virus is injected even if we ran back home today to -- all of us -- start working on our parts of this.

DR. JOHNSON: You said a magic word and that is "carcinogenesis." And I was wondering if Anne Pilaro would like to say a few words about AAV and carcinogenesis. I know that she is ready.

(Laughter.)

DR. PILARO: Actually I was not going to address AAV per se but more appropriately maybe for this audience is the timing of when carcinogenesis studies are expected and why you do them. What are the red flags that will do them.

(Slide.)

Thank you, Terry.

I just basically put together a few slides last night when I was thinking about this, realizing that probably the majority of this audience really does not understand or is familiar with carcinogenesis assays, why they are done, why the rodent bioassay is so important, and how it fits in with the field of gene transfer.

So what I really wanted to do is just give you sort of an overview and let you know that there are guidance documents available that tell you how to determine the need for carcinogenesis studies, how to select the dose to go forward in the study, and the timing of those studies, when they need to be conducted prior to what phase of clinical development you are in.

(Slide.)

The objectives or the purposes of doing the carcinogenesis studies are really to identify tumorigenic potential of an agent in animals before you go into large scale populations in the human. What these studies consist of is really a lifetime exposure from juvenile age of the animals, which is usually started about four to six

weeks of age, all the way through senescence. They are usually done for two years.

Doses are selected so that they are at or greater than human exposure. Now there is a guidance document on selection of doses that really applies to small molecular pharmaceuticals that tends to go for exposure levels based on human versus animal AUCs or pharmacokinetics data showing exposure.

That is not always feasible to do for the biologics and it probably will not be feasible to do for gene transfer vectors so what we really want to see is levels of exposure that would be approximately equivalent to or some magnitude greater than the human.

These are -- eventually when you get the data from these you want to do a risk assessment and be able to determine what is the relevant risk to humans out of the studies you have conducted.

(Slide.)

The need for carcinogenesis studies is defined in the ICHS1A document. The citation is down at the bottom. This is really determined by the intended clinical use and the document states that if the intended use is going to be continuous for at least six months you need to do carcinogenesis studies prior to marketing approval.

It is also expected that for use of products that will be six months or greater on an intermittent basis such as the agents for chronic rhinitis or for depression, some conditions like that, the carcinogenesis studies will also have to be available prior to marketing.

The other time that you would expect to see carcinogenesis studies is if the delivery system results in a prolonged exposure. For example, you have a depot mechanism where the drug is released slowly over time.

Now these documents, I will give you the caveat, they were written for small molecular pharmaceuticals but many of the principles do apply to biologics and to the gene transfer. We just have not seen enough of these products go far enough that we have really seen studies that become relevant.

(Slide.)

Now I mentioned there are a couple of red flags that are raised that will absolutely require you to do carcinogenesis studies. If your product has unequivocal genotoxicity when it is tested in the standard battery of mutagenesis assays, and that includes mouse lymphoma transformation assay, the AMES assay, bacterial mutagenesis assay, and the in vivo myconucleus assays, if you have unequivocal genotoxicity there, you will have to do carcinogenesis -- full carcinogenicity studies before you can even get started in Phase I clinical development.

Other red flags that raise what we call "cause for concern" are if you have a previous demonstration of carcinogenic potential in a related product in that same class or if the structure activity relationship of your product suggests there may be a risk, or if you have seen evidence of preneoplastic lesions in repeat dose toxicity studies that you have done up to that point in time in your clinical development program, of if you show that you have long-term tissue retention of the parent compound or of its metabolites that are leading to either some pathology or some change in the tissue where they are being retained.

These are what we call the "cause for concerns" that raise the red flag that says you need a carcinogenesis study before you can go further.

(Slide.)

The timing of the carcinogenesis studies is addressed in another ICH

document, the M3 nonclinical safety studies. This tells you when studies are expected prior to what phase of clinical development you are going to.

For carcinogenicity studies, they are not usually required prior to Phase I of development unless you hit one of those red flag cause for concern criteria. They are usually conducted concurrently with your pivotal or Phase III trials prior to marketing approval so that the data are available at the time that you have a licensing application submitted to the agency.

In some serious or life-threatening indications where there are no alternative therapies, these studies may be made as part of your post-marketing commitments or your post-approval studies. And in that case the lack of carcinogenicity data is addressed in the labeling for the product at the time of licensure. And these studies would be expected to be completed at some point in time after marketing.

(Slide.)

I just want to switch a little bit here and talk about some of the design of carcinogenicity studies and we do actually have a statistician sitting at the table who can maybe do some of the numbers for you guys and tell you what we are really looking at here.

But typically for small molecule pharmaceuticals, the carcinogenicity studies are done in two rodent species and it is usually rat and mouse. At minimum you expect to see 50 to 60 animals per sex per dose group treated.

And the reason I have "minimum" highlighted is frequently the upper dose levels may have significant toxicity and you see mortality. Once mortality drops so that you have maybe 40 percent of your starting animals remaining viable at the end of the study the validity of the assay comes in question so you really want to start with enough animals so that you have enough animals left at the end of the two year period to make a significant assessment of tumor frequency.

For dosing you want at least three dose levels, one of which is no effect level, in the toxicity studies. One which has some toxicity associated with it and one either at -- for small molecules the accepted level is 25-fold the human AUC by the pharmacokinetics assays or at a maximally tolerated dose that the animals -- a certain percentage of them will survive the treatment.

This is lifetime exposure. It begins very early. I mentioned earlier four to six weeks after birth. It continues typically daily dosing for the two year duration of the assay. At the end of the assay the animals are sacrificed. Every tissue is evaluated by gross pathology. Every tissue is weighed and they are evaluated histologically for evidence of tumors. So this is a very large, very resource intensive study.

Because of the recognition that there are limitations of these studies and because they do get very large and very resource intensive, there has been discussion about the use of alternative short-term models. For example, the p53 knockout mouse as alternatives to doing a full two-year bioassay. However, these assays are currently being validated and they have not been totally accepted for validation right now.

The current standard right now is that if an alternative assay is to be used, it should be followed up with a rodent two-year bioassay in just a single rodent species, either rat or mouse, whatever your dose ranging in your long-term toxicity study shows to be more sensitive.

And these parameters are outlined in the ICHS1B, Rodent Carcinogenicity Study, document.

(Slide.)

As it applies to gene therapeutics, what we have basically been working on for the past couple of years is how are we going to look at this when we are looking at the different vectors, and we have presented these next two slides at several different meetings, and I really want to just go over it now just to refresh everybody's memory and kind of lock in the next phase of the panel discussion.

When we look at the gene transfer agents, the standard rodent models and the two year carcinogenicity bioassay are probably not going to be appropriate. We already know daily administration of vector is not going to be feasible. However, we also know that several of these vectors, including AAV, continue to express over the lifetime of the animal.

the other thing that we have that may be a limiting factor is that the host immune response to the vector or to the transgene may either limit the toxicity or may have effects on tumor development. We may see enhanced tumor development because of inflammation or we may see suppression of tumor development because of activation of cytotoxic T effector cells.

So these are parameters that we have put out there for everybody to think about in development and what we have basically said in terms of carcinogenicity assessment for what we see right now in the gene therapeutic agents is that it will be product specific studies determined on an individualized basis whether they are needed or not. It will depend on the product class, the type of vector that is being used and the transgene that is included, the duration of clinical dosing and the intended schedule and route, the patient population, if you are going into adults or if you are going into children, and if you are going to be treating a serious and life-threatening disease as opposed to a less serious one with alternative therapies, and the risks of integration of the vector into the host tissue.

The dose selection will most likely be based on the maximally feasible amounts and on the amount of gene expression and reconstitution of the protein.

The need for many of these studies may be obviated by long-term clinical follow-up and we have had this discussion before the Biological Response Modifiers Committee about what constitutes long-term. My understanding is we are going to revisit that issue next month again and hopefully come out with some decisions on this.

So what we have really said here is that the typical carcinogenesis assays are not really going to be appropriate for gene transfer vectors. We may have to come up with alternative approaches and in the case of AAV we may not have those data available at the time of licensure but we will have to come up with a way to get them available very shortly afterwards.

So I just want to thank you for your time and actually I would welcome any questions, and turn this machine back over to Terry.

DR. JOHNSON: Any questions for Anne?

DR. SAMULSKI: Yes. Anne, I would like for you to -- first of all, I thought that was really fantastic and it gave us a clear picture. But based on where we are at with this preliminary data and everybody's interest in seeing it repeated, what is your comments from a regulatory point of view of direction you would encourage us to go in?

DR. PILARO: Well, we are going to get into that with the question three but my first comment was, "Okay, this is great. This is a finding. Let's see it repeated." And, you know, I guess I had the same feeling as everybody else's. Can

we repeat this? What was it due to? How are we going to tease this out?

And in looking at this we are going to get more into this in the discussion. There are a lot of variables here that we do not know could have contributed to the liver tumor formation.

One question that I -- I guess I can bring up now for Dr. Sands and for others is there was also a high level of expression of the gene that you showed in those immunohistochemistry pictures in other tissues, and did you ever look for gene integration there or vector copy number in there -- those tissues? You said they did not have any tumor formation.

DR. SANDS: Right. We looked -- we did not look for integration specifically but we looked -- we estimated copy number in a semi-quantitative PCR assay and the number of red cells that you see in a given tissue correlates pretty well with the relative level of AAV genome.

For example, the heart. The heart has an enormous amount of enzyme activity. It has quite a few positive cells also. And when you look at the relative amount of AAV genome there is a lot of genome in there.

In a tissue like the spleen there is very little enzyme activity associated with it. There is also almost undetectable amounts of AAV gene.

So it all correlates. There is genome all over the place.

DR. PILARO: Right. That is another point that we would want specifically to address in future studies is does the copy number or the amount of genome present correlate with enzyme expression and/or development of tumor.

So those are the kinds of questions that I had, Jude, when I first heard about these data so I think I am at least going along with the rest of the group.

DR. KAY: I may be jumping ahead but one thing -- at one point we had sponsored by the FDA a workshop on AAV issues and there were a lot of discussions at that workshop about what type of experiments to do with this particular problem, and there were so many variables, as you mentioned, that were raised and there are even more being brought out now. And whether it would be useful to resurrect that workshop before we go to the great expense and resource to repeat experiments without, you know, the right controls, et cetera.

DR. PILARO: Yes. I did not bring those slides with the mothership approach and the jigsaw puzzle approach. I apologize. I actually thought about that and went, no, that is getting out of hand here but we had a discussion about two years ago about ways to address specific questions, including insertional mutagenesis and the potential for carcinogenesis with AAV.

And at that discussion we decided that, you know, when you looked at all the different variables that had to be addressed, the different control groups that had to be addressed, and you actually did a power calculation based on the low incidence of tumor versus a high incidence of tumor, we were looking at anywhere between 4,000 and 18,000 mice, and that is part of the reason why the discussion got tabled at that point in time because nobody has got an animal facility that is that big and nobody would be able to do the study under the GLP conditions that would be required for this type of study through the agency.

However, there were points that came out of that discussion that probably should be addressed now and one of them that I can think of right off the top of my head is if it is really the virus a null vector should be able to show the same incidence of tumors as did the GUSB vector. So that is one thing we might want to consider exploring very early.

If it is really the animal strain then we should be able to take the normal background animals and the GUSB deficient animals, treat them both the same way and then see whether or not that comes up but I think that comes up in our third question so I will table that for now.

DR. JOHNSON: Let me just jump ahead a little bit. We have, you know, in essence, run out of time and I do not want to short-circuit the rest of the discussion because I think it is very important that we get to the next set of questions.

So for number three actually what I have suggested, and I think I have got reasonable consensus around the table, is that rather us sit here today, this afternoon in a matter of five minutes and try to design a set of experiments to answer either a very narrow range of questions or a very broad range of questions, I think that what I would recommend to Amy with consensus around the table is that we develop a smaller working group to be formed in short order to actually contribute to the design of the experiments that are designed under point number three.

I think for us to sit here this afternoon -- we could be here until midnight and never get to the panel II an Panel III. And, again given the time that it is already 2:30 and we are already behind, I think that makes the most sense. It is pretty clear that everybody agrees that the experiment needs to be repeated. It needs to be replicated and we need to see if it happens again but along with that some controls can be inserted.

Now, of course, there are caveats to that. As Mark points out, these mice are -- it is not like going to Charles River and buying 500, you know, mice. It just does not happen that way. So the experiments will have to be planned extraordinarily carefully and resources will have to be made available to do the experiments properly. Otherwise they are not worth doing.

So I think that unless I hear a stampede of protest, we would table number three to be taken up by a smaller working group to be formed very, very quickly.

Any other discussion on that?

Any other discussion on this particular issue?

Going once, going twice, gone.

So we are ready to move on to actually Session III now, which is "Clinical Studies Using AAV Vectors" and we are right back on time.

We need about two minutes to change people at the table here and get Pam Zeitlin up here. So if we could just stand in your place, stretch and sit back down, that will be sufficient.

(A brief break.)

## SESSION III: CLINICAL STUDIES USING AAV VECTORS

DR. JOHNSON: Next we have Pam Zeitlin from Hopkins who is going to talk about "Clinical Trials of AAV in Cystic Fibrosis patients: Initial Studies."

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CLINICAL TRIALS OF AAV IN CYSTIC FIBROSIS PATIENTS: INITIAL STUDIES

PAMELA ZEITLIN, M.D.

JOHNS HOPKINS UNIVERSITY

DR. ZEITLIN: And I want to make the point at the beginning that this was a two center trial.

(Slide.)

My colleague, Dr. Terry Flotte, who you heard from this morning has been conducting half of it at the University of Florida. And our sponsor is Targeted Genetics Corporation.

(Slide.)

Now this study was the first clinical study of an AAV vector. It was first reviewed and approved by the RAC in 1994.

(Slide.)

This is the vector. It is a very simple vector. ITRs, the CFTR full-length cDNA, the poly-A and then another ITR. So you can see right away that the promoter activity is coming solely from the ITR, which provides relatively weak promoter activity.

(Slide.)

Now the study objectives were first to assess the safety of single dose administration of TG/AAV/CF to the nasal epithelium and to the superior segment of the right lower lobe. Assessment of gene transfer expression and biologic activity were to be made. We wanted to assess the clinical impact of a single dose of vector gene transfer and to evaluate the immune response.

(Slide.)

The study design was dose escalation and it was applied to one nares with vehicle solution to the opposite nares in a double blinded placebo controlled randomized fashion and an open label administration to that segment of the lung lobe that I spoke of. The doses here -- we started out very low as you can see -- $6X10^4$ DRP up to $2X10^{11}$ DRP in nasal epithelium with the lung dose being $6X10^4$ up to $2X10^{12}$ DRP. We had two patients per cohort with a total of 25 patients.

(Slide.)

Now I can barely read this on the screen of my computer but these are the serious adverse events during the trial and, first of all, you can see that the most common one up at the top is pulmonary exacerbation. Pulmonary exacerbations are very common in cystic fibrosis. These are adult patients and they tend to have one or more per year. You can see that they occurred at all dose levels here.

This was followed by hemoptyses. This was mild and not severe.

One episode of pneumonia. That is the third word down there. Pneumonia.

Then we had three events of sinusitis. Sinusitis is very common in cystic fibrosis, 99.9 percent of people with CF have sinusitis from the moment they are born practically.

There were two headaches.

Some rhinitis. One of our subjects eventually -- it was revealed that he had a drug addiction. We do not feel that is related to the study vector.

And then some back pain.

(Slide.)

And we continue with chronic sinus disease, migraine. Two migraines

in one patient. One abdominal pain. One appendicitis. Appendicitis is a frequent complication that is seen in cystic fibrosis from the gastrointestinal manifestations of the disease. And one episode of atrial fibrillation. And these were felt not to be related to study drug.

(Slide.)

So our results -- we feel -- the way -- at the doses given, in the manner that they are given to this particular study population that the single dose was generally safe and well tolerated. There were a total of 39 serious adverse events in 12 patients out of 25. Only one of them was thought possibly related to vector administration.

And this was a pulmonary exacerbation that occurred seven days post-vector administration in one of the high dose cohorts, $2X10^{12}$ DRP open label in one lung lobe. The patient actually did not really feel sick but because of a drop in pulmonary function there was a semi-elective admission ten days later for IV antibiotics and he recovered without sequelae. There had been a prior history in this subject of three similar exacerbations in the preceding six months.

(Slide.)

So to summarize to date, we did not really talk about the first study, 9501, that was a sinus administration of this vector to 33 CF patients. Then I reported to you 9502, which was the both nose and lung administration in 25 patients.

And there has been a study, 9701, of aerosolized lung administration in 12 patients and we have actually started at some sites, 25B01, a multidose aerosolized lung administration with 36 planned.

So I could almost say that we have almost dosed more human patients with this particular vector than mice with some of the other vectors that we have been discussing.

(Slide.)

Now I know that you cannot read this and I apologize but this is the aggregate safety data from all of the different trials. So the first column is the sinus trial, 9501, then my trial, then the 9701 to date, and so in all trials N=70 here. Thirty-five events in 25 patients or 36 percent reported a pulmonary exacerbation some time after vector administration. There were six events in four patients or six percent of hemoptyses. Three events in three patients or four percent of pneumonia and then the percentages of each of those other things that I talked about like headache, rhinorrhea, migraine, abdominal pain were all about one percent. There was urticaria down at the bottom that occurred in the sinus trial. Just one episode even of urticaria.

(Slide.)

So 70 patients to date with CF have been administered this vector to the sinus, nose and lungs. We feel that it is generally safe and well tolerated and that the SAE profile is consistent with the underlying CF disease.

And that is what I had to present. I can take questions.

(Applause.)

DR. JOHNSON: Thank you, Pam.

Questions for Pam?

DR. _____: (Not at microphone.) (Inaudible).

DR. JOHNSON: Can you repeat the question?

DR. ZEITLIN: I did not hear the whole question.

DR. _____: I guess the question really relates to biodistribution, of course, because all your side effect profiles focused on the

respiratory system apart from -- I guess you were just looking at global symptoms. But in terms of more detailed evaluation, in terms of liver function tests, in terms of particularly what we know retrospectively, of course, with your high dose distribution with intranasal delivery, for example, one has presumably quite reasonable potential for uptake into the vascular system and did you actually look all over the body? What was the biodistribution? Did you look in any of those target organs in those clinical trials?

DR. ZEITLIN: In these particular subjects I believe Targeted Genetics has the blood. I have not seen all that data. Maybe Dr. Tom Reynolds might want to comment on that. We did not see any dissemination of vector is my summary of those results.

DR. REYNOLDS: Yes, Pam, I will try to comment off the cuff. We were asked not to bring data today so my recollection is we did test the blood of all patients that have received vector. We have one patient from the aerosol trial at the lowest aerosol dose that had a positive PCR signal in the blood at the limit of detection. All the other patients have tested negative for vector in the blood.

As you know, we try not to do invasive sampling on patients in areas that we are not aware of problems so we have done no liver biopsies on any patients. However, there is one patient that did receive vector at Stanford in the sinus study who subsequently died of a cardiovascular event after he was off study. An autopsy was performed and there was no vector present in the liver of that patient.

DR. JOHNSON: Over here?

DR. MARKERT: Louise Markert, RAC.

I was just curious that of your 25 patients, three of the CF patients had hemoptyses, one of which was life-threatening. I mean, maybe it is because I am a pediatrician and I do not tend to think of hemoptyses as being in three out of 25 patients over a short period of time. Are these very late stage patients?

DR. ZEITLIN: I believe that these must have been related to the bronchoscopy procedure, which is extended in length in order to lavage and do the brushings to get the cells that we needed to do for the PCR. So I believe they were felt related to the procedure and the underlying disease rather than to the actual vector.

Now, hemoptyses, just to answer your general question, hemoptyses is a relatively frequent complication in the older CF patient that is felt related to the dilatation of the bronchial vessels supplying the very inflamed and bronchiectatic lung.

And so any little trigger like a superimposed pulmonary exacerbation or perhaps a vigorous brushing would then expose those vessels to bleeding.

DR. _____: (Not at microphone.) (Inaudible).

DR. BREAKEFIELD: Please come to a microphone.

DR. JOHNSON: A microphone, please. Come up here to the table.

DR. BREAKEFIELD: Thank you.

DR. FLOTTE: So the one subject at the University of Florida who had what was considered life-threatening because he required embolization, in that case the hemoptyses event was observed directly to be occurring at the time of the first brushing and so this was -- this was a directly observed event, which is something -- you know, is a complication of -- it is a known complication of this procedure in patients who have this problem.

It was unfortunate but the patient is fully recovered and no longer has this -- you know, has recurring hemoptyses. But, you know, it was clearly

instrumentation related.

DR. JOHNSON: Xandra?

DR. BREAKEFIELD: I assume these patients are still being followed so if they actually do get some kind of lung cancer we will find out about it?

DR. ZEITLIN: Yes. These patients are going to be --

DR. BREAKEFIELD: How long have they been followed?

DR. ZEITLIN: -- undergoing annual follow-up for the rest of their lives and our's.

So we make a contact. We have a series of questions and they report back to us and we report it to the NIH and everyone else.

DR. BREAKEFIELD: I did not remember.

How long have some of these -- has it been since some of these patients received the vector?

DR. ZEITLIN: Yes. For this first trial it was actually quite a lengthy trial. I think it spanned at least two years because we started at very low dose cohorts. So some people are two years out or more.

DR. FLOTTE: '95.

DR. ZEITLIN: '95. The first patient was '95. I have lost track of the time. And they still -- we do see them.

Some of them are in our own clinic. They are our local patients. Some of them are from the rest of the East Coast. And the most recent series of the higher dose cohorts were finished up this summer and we have not seen any lung tumors.

DR. JOHNSON: Any other questions for Pam?

If not, let's move on to the next. "Clinical Trials of AAV in Cystic Fibrosis Patients: Subsequent Studies" by Phyllis Gardner.

CLINICAL TRIALS OF AAV IN
CYSTIC FIBROSIS PATIENTS:
SUBSEQUENT STUDIES
PHYLLIS GARDNER, M.D.
STANFORD UNIVERSITY SCHOOL OF MEDICINE

(Slide.)

DR. GARDNER: The trial that I am going to be presenting really is, of course, the work of multiple people, probably the least of which is me even though I was the principal investigator. So I just want to acknowledge the fact that several Stanford colleagues took part in this, including Rick Moss, who runs the pediatric clinic, John Wagner, who was my fellow, who is now at Merck, and Mary Lynn Moran, who is the ENT surgeon.

We used the vector developed by Terry Flotte and Pam Zeitlin and others at Hopkins, Bill Giggino.

We worked with Targeted Genetics and so they provided the vector and a lot of the clinical analysis with us.

It was sponsored by NIH GCRC money as well as CFRI, Cystic Fibrosis Research Incorporated, a small foundation in the Bay area.

So it is a multiple group study, NIH sponsored.

(Slide.)

So this is a two-stage study, Phase I and II. You have heard some of the results already with respect to the serious adverse events, of which there were none.

(Slide.)

So we did this trial in the maxillary sinus and we did this -- we hypothesized that the sinus would be a good surrogate model for lung disease for a safer method to approach the question of whether AAV CFTR could safely and effectively transduce CFTR.

And the reason the sinus, we thought, would be a good model is that you have -- as Pam Zeitlin mentioned -- virtually all patients because the airway epithelium that line the sinus are also affected in the disease cystic fibrosis, you have radiographic panopacification of sinuses in 90 to 100 percent of patients.

They have a sinusitis that has pseudomonas -- often has pseudomonas colonization and then inflammation and infection, subsequent inflammation and infection. And it mimics the same disease that you see then, colonization and disease, you see in the lower airway tract.

At Stanford we have for quite a long time done a procedure called a bilateral antrostomy to correct the disease in the sinusitis. That is that the ethmoid sinus is surgically operated on and the maxillary antrum is widened so that one can have access into the sinuses with a catheter to perfuse the sinuses with tobramycin.

Now it turns out that not all people in the country believe in this particular approach but the people in the Stanford area certainly believe in it because patients are very affected by their sinus disease. They have chronic headaches and all the other things that go along with sinusitis.

It was started because we had a big transplant population and the trans -- what they found with the transplants at Stanford, their observation was that they -- because the sinuses are colonized with pseudomonas that they had -- and because your patients after transplantation with their lung are immunosuppressed that there is a higher incidence of pneumonitis in those patients. So they started clearing out the

sinuses prior to transplantation and then a whole cadre of patients came forward because they found that they were so relieved by the procedure.

So we have a large group of patients over quite a few years who have developed -- who have had this procedure and it -- we thought it would form a very nice -- it was Jeff Wein actually who first came up with this.

(Slide.)

Jeff Wein is in psychology at Stanford. He said, "This would be a perfect inpatient control. Easily accessible site, small, affected by the disease, and you could have it inpatient control." So that is why we chose the maxillary sinus as the site for installation of vector and proposed that possibly it could be a good surrogate model for lung administration.

And as you can see there, this is a patient who has had the antrum widened and a catheter is inserted in there. You can tape it in and it can stay in there. And fluid can be instilled and biopsies can be taken. We also can endoscope it and see the whole sinus so it is a reasonably easy model that is relatively safe for the -- well, very safe for the patient. Not always comfortable but safe.

(Slide.)

So the same vector that Dr. Zeitlin described was used, that is once again the Targeted Genetics CFTR AAV vector and it has just the ITR regions working as the endogenous promoter. CFTR cDNA and the poly-A marker. And it is a weak promoter.

(Slide.)

Now that vector was used in two different trials. This is the 9501 trial that we were talking about and I think it also dates back. We have lost track of time. Almost to '95-6. Around the same time. So the first patients who saw this vector in these trials, it has been about five to six years. It is very hard to believe.

So the first trial was a dose escalation to one sinus open label. The dose levels were $10^2$ to $10^5$ replication units, which is equivalent as you can see to the DRP equivalents. There were ten patients who were treated, 15 sinuses. That means five of the patients went -- underwent repeat procedure. And they had -- all had chronic sinusitis with cystic fibrosis. They were all correctly identified and they all required bilateral antrostomies. And we had major endpoints, safety, gene transfer and electrophysiology.

(Slide.)

One of the interesting things to us was that the repeat -- the -- we were able then in the repeats to observe whether or not they developed neutralizing antibodies for a second administration. We were also able to look and see if they still had transgene expressed.

The maxillary sinus Phase I results, we found that it was very safe and well tolerated. There were no serious adverse events. We will present the ones -- aggregated SAEs for both trials later. There were no inflammatory responses at all. We did not see any evidence that -- like with adenovirus in the CF trials, we did not see evidence of an inflammatory response to vector administration. And we saw no evidence of neutralizing antibody titer change. So it appeared to be tolerated, not to have an immune response or an inherent inflammatory response as the vector alone, and safe.

(Slide.)

We did semiquantitative DNA PCR and biopsies and it appeared that we had transgene expression that was dose dependent. We got 0.1 to one vector copy

per cell at the highest dose, $10^5$ replication units per ml. So that was the dose chosen for the second trial and it had a pretty nice dose correlation.

(Slide.)

We also found in that particular trial we did transepithelial potential differences. Now this is not easy in the sinus. We managed -- John Wagner did this. And what you can see down there is at the upper two doses, 50,000 replication units and 100,000 replication units per cell, that it appeared we had a significant change in transepithelial potential difference as if the gene that was being expressed was correcting the underlying defect. That is to say we normalized the transepithelial potential difference in those treated cells.

This was measured at 14 days out and we -- I should say we also saw evidence in that trial of expression 90 days out but we did not do all of the transepithelial potential differences all at that time. They are complicated.

(Slide.)

So on a Phase I trial it appeared to be safe. We established the dose of $10^5$ replication units and we had evidence of functional expression of the gene.

(Slide.)

Well, I just said that, didn't I?

We did see persistence for about 70 days and up to one gene copy per cell achieved. We had also seen repeat delivery at high dose was well tolerated.

(Slide.)

We went on to a maxillary sinus Phase II trial. That became a randomized double blind placebo controlled trial in which the patient served as its own control. So on a randomized fashion we administered vector to one sinus and placebo to the other. And the dose was $10^5$ replication units. There were 23 patients and the patient population was the same as Phase I but we allowed the age to go down to 15 years or greater.

(Slide.)

Some of the patients who participated in the first trial also participated in the second trials. In terms of safety findings, vector treatment produced no consistent change in neutralizing antibody titer or sinus histopathology. Once again in following up neutrophil counts, biopsies, neutralizing antibody titers, we found no significant effect of vector, which is not to say that these sinuses are not intensely inflamed in and of themselves.

We saw no adverse respiratory events related to the vector but there was one possible related -- what was deemed as a nonserious acute vestibulopathy. Ear pain, I think.

(Laughter.)

(Slide.)

The sinusitis -- now in terms of the effects, so this was a Phase II trial to try to determine efficacy. Here is where our surrogate model, I think, failed. We -- there is really not a good way to assess efficacy of treatment in the sinusitis model. So we were making it up on the fly what we would follow and we tried to determine parameters to define what sinusitis was. And they were made up. They were clinical parameters.

We found that it recurred at least once in 18 of the 23 patients 78 percent of the time and did not appear to be related to vector versus placebo. The recurrence and the time to recurrence were statistically undistinguishable, therefore, between the two groups.

(Slide.)

We did see, though, some evidence for some effect and I am going to jump ahead because on another slide it will say this but one of the things that confounds that study in retrospect is that we did not withhold tobramycin -- intravenous tobramycin therapy from these patients and they are a generally sick group who often have pulmonary exacerbations and for pulmonary exacerbations they get tobramycin, intravenous tobramycin.

So while they were not getting tobramycin instilled in their sinuses over the three month period of the trial, the 90 day period, they were getting -- almost all the patients had some point in time intravenous tobramycin and that was not randomized by the patient population because we were -- we had to treat the patients when they had pulmonary exacerbations. So it was a confounding factor that we just could not deal with retrospectively.

One thing that we found interesting -- Dr. Rick Moss at Stanford has been following, as with many others, cytokine levels. And he finds differences in pro and anti-inflammatory cytokines. And we felt we found a statistically significant effect on cytokine expression. In vector treated sinuses cytokine -- the pro-inflammatory interleukin-8 decreased from study day zero to day 14 by 1,475 picograms per ml as compared to 353 in the control trial. And, of course, they had tobramycin just prior to this. So the control might have -- might be reflecting the effect of tobramycin but there was a greater decrease in the pro-inflammatory IL-8 in the vector treated sinuses and that was statistically significant in the one tail T test for each group.

(Slide.)

And if you looked at day 90, the vector treated group had an increase in the pro-inflammatory IL-8 while the vector treated group was normalizing back to about baseline.

(Slide.)

So we interpreted that to say that the AAV CFTR did not alter sinusitis recurrence. And, as I said, antibiotics may have confounded that outcome but the pro-inflammatory cytokine IL-8 decreased after AAV CFTR treatment, suggesting that a vector treatment related to a decrease in sinus inflammation.

In addition, we saw statistically significant, by the single tail T test, changes in the anti-inflammatory IL-10 that would be what you would predict. We saw increases in IL-10 expression in the vector treated versus the control. So the cytokine expression gave some indication that possibly we had an efficacious gene transfer.

(Slide.)

I will also go on to say that for reasons that we cannot possibly interpret, though we tried desperately, repeating everything, we could not get interpretable DNA, semiquantitated DNA PCR results in our biopsies so we have no idea by test whether or not the vector was expressing and these were the same tests that we used in the Phase I trial and it just highlights how difficult it is to do some of these trials.

(Slide.)

Now in terms of aggregated secondary adverse -- I mean, serious adverse events, we had, as usual, the pulmonary exacerbations, asthma, cholelithiasis, and urticaria. One event of that.

(Slide.)

Here is the total which you are not interested in because --

(Slide.)

But anyway the bottom line was that none of these seemed to be in any way related to the study agent and they were -- most of them were consistent with the underlying disease.

(Slide.)

So, in summary, the vector gene transgene construct was well tolerated with no serious adverse events. There was persistent and efficient gene transfer observed in the Phase I trial at least and evidence in the Phase II trial by changes in cytokine levels as well as the transepithelial potential difference in the Phase I trial.

We had evidence of vector derived CFTR expression. It was based on this that a randomized aerosolized -- I mean, a dose -- the next aerosolized trial was initiated. That is a multicenter trial. There was first the dosing study and now it is moving -- it was going -- it has moved into the randomized trials because we felt at least in our surrogate model, if not perfect, we had felt reasonably happy to move forward in terms of safety of this underlying experimental therapy.

Thank you.

(Applause.)

DR. JOHNSON: I think Phyllis can take a few questions.

Mark?

DR. KAY: Phyllis, is there any way that you have been able to differentiate the double stranded genomes from single stranded genomes by this PCR assay because the copy number is pretty high and I am trying to get an idea of how much of it is actually double stranded. I know it is probably difficult to do.

DR. GARDNER: There is certainly nothing I did that could distinguish it. I could look to Terry to ask him if you have any evidence.

DR. FLOTTE: Targeted Genetics did the assays.

DR. JOHNSON: Microphone, Terry.

DR. FLOTTE: Sorry.

DR. JOHNSON: Why don't you just move back to the table?

DR. FLOTTE: Tom would be the person to comment on the semiquantitated PCR on the clinical samples. Those were done there.

What I will tell you is that in the preclinical studies in the monkeys and the rabbits, Southern blots show double stranded forms at the time points when we are harvesting, which are out, you know, three to six months.

But Tom can answer.

DR. GARDNER: Any other questions?

As I said, these are patients now. Oh, yes, Tom?

DR. JOHNSON: Tom?

DR. REYNOLDS: So for the sinus trial, as you can imagine, the biopsy specimens that were taken by our investigators are taken by forceps biopsy and they are just directly kind of pinched off from the inside of the sinus. There are chunks of tissue that are about 10 to 20 milligrams. So they are a very small number of cells.

The only technology that we could apply to them to quantitate the amount of DNA was PCR. From the way our vector is constructed, it is really not possible for us by PCR technology to distinguish single and double strands, and we did not have enough DNA really to do a Southern blot so we do not have that.

The other point that I would make about the vector copy number

relative to the dose is the sinuses in these patients have a space that is very, very small because they are nearly filled in with fibrotic tissue. They are about one centimeter volume total. One cubic centimeter.

And so as a result there is pretty high local concentration of vector in that epithelium and I do not -- and we are pretty comfortable that we could be seeing a gene transfer with a copy number of about .1 to one copy per diploid genome in that small sinus cavity.

DR. JOHNSON: A question here at the table?

DR. JUENGST: Could you explain again about the pulmonary exacerbation? Why that is not associated with the vector?

DR. GARDNER: Sinus. These are once again CF affected patients, some of whom have relatively severe disease, so that level of pulmonary exacerbation --

DR. JUENGST: Just the course of the disease.

DR. GARDNER: -- is the course of the disease and did not appear to be different between the two groups.

DR. JOHNSON: Ted?

DR. FRIEDMANN: Could you just repeat or remind us the differences or similarities between the structure of the epithelial surface in the sinus compared with upper airway? And, also, on what basis you included in your summary slide the presumption that you were getting gene expression?

DR. GARDNER: Right. The up -- as I understand it, and I certainly am not the expert on this, but the epithelium that lines the sinuses, it has -- it is a respiratory, secretory epithelium that expresses CFTR that has chronic pseudomonas colonization and has an appearance of pathogenicity that mimics lower airway disease.

Having said that, it also has a difference in the transepithelial potential difference being hyperpolarized relative to normal tissue. Having said that, I am sure there are differences on a finer tuned level in terms of how much are secretory cells -- scilliated secretory cells versus goblet cells versus basal cells and other parts that are finer nuances that may not at all be the same.

I am sure that the nasal and the sinus and the upper airway you would probably start to see transitions in those. Now in terms of why do we think that we had gene transfer in the Phase I study, it was through the semiquantitated PCR and the transepithelial potential difference. I think it is weaker by far in the Phase II studies since our DNA PCR results just were -- they were just completely uninterpretable.

And the only thing that we had to suggest it was the alterations in a statistically significant fashion in vector versus controls in cytokine levels, in two different cytokine levels, and that is all we have. And it is -- and I would be the first to say that is only just suggestive possibly.

DR. FRIEDMANN: It is better than nothing but it really does not --

DR. GARDNER: It does not --

DR. FRIEDMANN: -- speak to the CFTR.

DR. GARDNER: I would not go out and tell you that we cured their sinuses by any stretch yet. I just think it was enough for us to say this is safe enough to go on and aerosolize and try it in the lung, which is the primary model that you are trying to treat.

DR. FRIEDMANN: So it is a safety comment rather than a gene expression comment.

DR. GARDNER: Safety comment with the caveat that possibly. One

of the other things that we -- and we have submitted this, it is in review -- one of the other suggestions we make is that possibly we should be studying as an outcome, a secondary outcome, cytokine parameters because it is an earlier assessment.

And, in fact, we looked back at Robert Altman's studies and he had noted in his studies alterations in cytokine levels, and those were both nasal and airway administration, and he put it in the -- I think he put his results in the methods section but he saw an alteration in vector versus placebo control in cytokine levels as well and pro-inflammatory cytokines being decreased by vector administration.

So we thought it was an interesting observation that if you look back in the literature someone else had found and thought that perhaps this could be a secondary outcome that people would follow and, in fact, we are following in our aerosolized trials.

DR. FLOTTE: Can I just comment on that?

DR. JOHNSON: Terry, a microphone.

DR. FLOTTE: It is really not necessarily that secondary in terms of -- perhaps some people have not followed the CF story recently. It is quite clear that there is a primary abnormality. I am not telling you anything you do not know but primary abnormality in cytokine gene regulation related to the CFTR defect, which probably represents a cell stress response to DF508 CFTR in the endoplasm particulum. But be that as it may, in primary cells one can demonstrate in a fashion that is directly related to CFTR expression that CF defective cells have in response to pseudomonas or bacterial LPS an exaggerated IL-10 response in deficient -- I am sorry, an exaggerated IL-8 response and deficient IL-10 response.

So it really is felt that the cytokine profiles represent a primary effect of CFTR that the relationship of which to chloride transport is not entirely clear and it may not exist at all. So this -- I would say that you are looking at two different aspects that may be important and fairly proximal to the CFTR defect.

DR. GARDNER: Yes. And I would stress that I just did a lecture on CF and I was talking about how Lou Stryer used to say, "One gene, one defect," and that confounded, I think, the whole field of CF for a long time because people kept trying to relate everything back to chloride transport and it is clear that multiple cellular functions are disrupted probably because there is endocytic recycling of a complex of proteins that are coregulated with CFTR and a variety of other things.

The other thing is that we found evidence, and it caused me to have mud and eggs slung at me for years, but we found CFTR expressed in lymphocytes. Pretty clearly it is expressed in lymphocytes and it -- what the physiology of that, the pathophysiological consequence of that is not clear but I would also just say one caveat. One has to remember that there are other cells in there in the sinuses. Some of them are cytokine expressing, as are the secretory epithelial cells, and they have misregulation as well.

So one does not want to over simplify the model.

DR. JOHNSON: One final question.

DR. WEISS: I just want to clarify the potential confounding effect of IV tobramycin. Was everybody more or less on IV tobramycin during the study?

DR. GARDNER: There were 19 pulmonary exacerbations and of those 19 they all got IV tobramycin. And I do not remember the exact breakdown of where they were but it was relatively similar in both. And it will certainly change the outcome of your sinusitis. I mean, IV tobramycin will treat your sinusitis quite well. It is just that we do not usually do that.

DR. WEISS: Except that 78 percent or so of patients had an additional episode of sinusitis.

DR. GARDNER: Well, the sinusitis was no different between the two at the same time they were -- there were 23 patients, 19 episodes of pulmonary exacerbation. Right? So that is a pretty high percentage of them that had IV tobramycin at variable times. But between the vector and the control group it was not as if there was a big dispersion and when they got it or whatever. It certainly would be a confounding factor in looking at sinusitis recurrence because while we withheld local treatment for sinusitis, we could not withhold the systemic treatment.

DR. JOHNSON: Great. Let's move on to the second clinical application currently in progress, "Clinical Trials of AAV in Hemophilia B." Bert Glader from Stanford University.

CLINICAL TRIALS OF AAV IN HEMOPHILIA B

BERTIL GLADER, M.D., Ph.D.

STANFORD UNIVERSITY

DR. GLADER: Thank you. It is a pleasure to be here.

(Slide.)

I would like to start out by making a couple of remarks about hemophilia for the ten people in this room who do not know about it.

Hemophilia is the hereditary deficiency of protein necessary for coagulation. I am sure you cannot even see this in the back. This is the coagulation cascade and when we talk about hemophilia, virtually every factor has been -- deficiency in every factor has been described but for practical purposes, Factor VIII in classical hemophilia and Factor IX are the major entities that we are interested in.

Approximately 85 percent of cases are due to Factor VIII deficiency and the remainder are due to Factor IX.

(Slide.)

Pediatricians are always accused of showing pictures of kids so I am not going to disappoint you. This is a colleague of mine. Some people in here may know Dr. Laurie Namen. Taken many years ago when he was a resident with his patient, Steven Christmas. He recently gave this to me and it just brings this all back to reality. Steven Christmas was the first patient described with hemophilia B.

(Slide.)

Now when we talk about hemophilia, we also classify it based on severity. Either calling it severe, moderate or mild, and that severity is based on measured factor levels. Severe being zero to one percent of normal, moderate is one to five percent of normal, and mild is somewhere five to 20 percent activity.

But this classification also is borne out clinically. It correlates with the bleeding tendency. People with severe hemophilia, less than one percent activity, have spontaneous bleeding without trauma. Often 20 to 50 bleeds a year occurring in joints and other soft tissues.

In contrast, people with mild hemophilia have one to five percent activity. Not much of a difference. However, their whole life their phenotype is entirely different and most of them only bleed with significant trauma.

We now also know from prophylactic studies done in children if you can transfuse them with protein concentrate and at all times maintain them at a level just above one percent factor level, you can prevent all joint bleeds. If they have joint disease you can arrest the progression of that.

(Slide.)

So because we know you need very little here, this has been a goal of gene therapy and because we know prophylaxis keeping people at one percent level will prevent a lot of these complications, this has been very exciting.

So based on a number of years of studies in animal models, both in mice and dogs, animal models for hemophilia by several distinguished scientists here, we have -- the safety and efficacy of AAV gene therapy has been documented and based on that a clinical trial based on all those studies, efficacy and safety, a clinical trial was started entitled, "A Phase I Trial of AAV Mediated Muscle Directed Gene Therapy for Hemophilia B."

This study was led by Dr. Katie Manno and Kathy High, and colleague Roland Herzog at Children's Hospital of Philadelphia. Mark Kay and myself were involved at Stanford. Alan McClellan and Linda Kudo were our colleagues at Avigen. And a variety of other individuals and institutions also participated in providing us patients.

(Slide.)

Now the specific aims of this study were to test the hypothesis that AAV mediated muscle directed gene transfer is safe and can result in an improvement in the clinical course of hemophilia patients without adverse events.

Secondly, we wished to characterize the human immune response to the transgene product and to its vector.

And, three, to determine whether germ line transmission of the vector occurred following IM administration.

(Slide.)

The study design was an open labeled dose escalation safety trial of AAV human Factor IX administration by IM injection. Three subjects in three different groups receiving a low, medium and high dose were to be entered. The clinical protocols were reviewed and approved by the local IRBs and IBCs, as well as the FDA and OBA.

The intramuscular injections of factor occurred under coverage of Factor IX concentrate so patients would not bleed and a variety of serum chemistries and blood studies and Factor IX levels were done throughout the study. And also studies to monitor for the development of inhibitors. And, lastly, muscle biopsies were looked at also for evidence of gene transfer.

(Slide.)

Now the inclusion criteria for this study was adult males with severe hemophilia B and no history of inhibitors to Factor IX. Inhibitors occur in 20 percent of patients with hemophilia A much more common but they are much less common in Factor IX deficiency. But they -- once you have an inhibitor it makes managing bleeding episodes much, much more difficult. And so we did not want an inhibitor because, in particular, we were concerned with the possibility inhibitors could develop with gene therapy gene approach.

One of the criteria here was that there had to be more than 20 days exposure to Factor IX concentrates without evidence of inhibitor. Now that is because almost all inhibitors develop within that time frame. In reality that has not been a problem because most of our adults have had hundreds of exposures and so -- and they have not had inhibitors.

And life expectancy less than one year was an issue so that they could complete the study and also the presence of a missense mutation because the inhibitor development has been associated primarily with large lesions and I think there is only

one or two cases of inhibitors developing in patients in Factor IX deficiency who have missense mutations.

(Slide.)

The exclusion criteria were any acute infectious disease going on, any end stage renal or liver disease, and we will talk a little bit about this in a second, any inflammatory bowel disease at the time when they would present, thrombocytopenia less than 50,000. Someone who is unwilling to stop prophylaxis regimen -- the prophylaxis regime. Many patients treat themselves prophylactically two to three times a week indefinitely to prevent bleeding and obviously we could not have somebody on that if we were going to test the efficacy of our gene.

(Slide.)

Okay. The subjects studied, we had eight subjects ranging in age from 23 to 67 years of age, who have been entered on study. The race is seen here in the second line. Every one of them was a severe hemophiliac by definition of having less than one percent activity and their CRM, cross reacting material, status was negative to positive, meaning they may have had some residual nonfunctioning protein.

(Slide.)

One of the major problems in hemophilia has been the exposure to blood products which has led to obviously HIV infection and, in fact, hepatitis C infection as well. And the vast majority of adults with hemophilia are, in fact, hepatitis C antibody positive and you can see here that six of our patients were antibody -- hepatitis C positive, two were also hepatitis B and two were HIV positive.

(Slide.)

The doses: To date, eight patients have been entered in three different levels of dose. The doses predicted -- were calculated based on the -- what was expected from the mice and the dog data. And at the low levels no results -- it was not anticipated there would be any effect and only at the higher levels would you approach anything near one percent.

(Slide.)

You can see the dose per site was the same. The reason for that being that there was concern with inhibitor development if there was a bigger concentration of dose at any one site. Because of that as the dose increased you had to increase the number of sites and the patients receiving the highest dose to date have had up to 70 to 100 injections into their muscle.

(Slide.)

The vector is administered in the CRC either at CHOP or at Stanford and prior to that they get 100 percent correction of Factor IX concentrate and they will not bleed with that. They are given local anesthesia and conscious sedation is necessary.

(Slide.)

Tattoo marks are applied to the injection site so that one can come back for a biopsy and then the multiple injections are used initially in mainly the vastus lateralis muscle and subsequently we have to move from there. The patients are routinely kept in isolation for 24 hours.

(Slide.)

This is just a picture of one of our surgeons and colleagues, Dr. Eric Scarsgard in the process of injecting the vector under ultrasound guidance.

(Slide.)

Okay. Adverse events to date related to the procedure: Mild swelling,

tenderness and some pain at the vector injection site. This is seen in six subjects at all doses. It was not significant but it did exist. Small hematomas and bruising at the vector sites was reported in five subjects and again at all doses.

In one patient they actually changed the tattoo material from methylene blue to India Ink and the patient had a fairly significant reaction to it so it was obviously a reaction to this and to the procedure but not to the vector.

And in one patient there was a transient fall in the platelet count to 110,000. He had had that before and so that was not a new event that it had fallen from normal to that level.

(Slide.)

Okay. So in terms of looking at efficacy, muscle biopsies were done after a couple of months and what one sees here is an H&E stain showing no evidence of any inflammation. This is typical of early patients and seen throughout. This is a stain immunoperoxidase for Factor IX in normal muscle. Normally you see no Factor IX there and here is a Factor IX from one of our patients and you can see that there is Factor IX expressed in that muscle biopsy.

(Slide.)

This slide summarizes the eight patients to date looking at either their Factor IX immunohistochemistry as I just showed you. Mostly positive in most patients. Looking at PCR and Southern blot for muscle biopsy and also getting evidence of gene expression. In terms of the level of circulating factor which we have achieved, the first patient actually -- it was less than one percent at the beginning -- got up to over 1.6 and subsequently has had a decrease in his Factor IX infusion to about 50 percent of what he used the previous six months.

In the second patient we had a level of .8 percent and that is varied between .6 and .8, and in the first few months after infusion he had an 80 percent reduction in his factor usage but this is also decreased now. He is off study in the year and he is -- but still he is using about 50 percent of what he used the preceding six months before going in the study.

None of the other patients seemed to have any significant rise in their factor level and the remaining patients here, one of them has a slight decrease -- Patient F -- in his factor usage. Three of the others do not and we are still -- the last couple of patients we are still pending more information.

(Slide.)

What were the safety issues we were concerned with here? First of all, inhibitor formation, then germ line transmission, and obviously insertional mutagenesis. The inhibitors we looked at -- there are different ways of looking at inhibitors. A functional test and so-called Bethesda assay is done periodically throughout the course of the study here and N/D seen across the board here for all of them means not detected. So we have not detected any Factor IX antibodies by the Bethesda assay.

(Slide.)

And looking in a more -- looking for also noninhibitory antibodies and Western blot. This is a positive control here. And looking at all our patients, actually A through G at different periods, they were all negative.

So I think we can conclude from this we have no antibodies that have developed to date.

(Slide.)

The risk of germ line transmission, obviously there is a concern the

vector sequences could be passed to progeny and we do not know what effect that might have on the fetus. Our plan for going into the study was we obviously were going to monitor for presence of vector sequences in the semen and we encouraged barrier contraception until the semen was documented negative at least on three occasions. And we also encouraged all patients to bank sperm until the sperm was shown to be negative.

(Slide.)

This is the data on PCR data and vector shedding, which was done, and you can see here for stool, urine, serum, saliva and semen. The stars here show what was positive and you can see that in the urine in a couple of patients on day one and the serum on day two for four patients, vector sequences could be detected. Beyond that there was no evidence of any vector shedding and also at no time in the study was there any evidence of vector in the semen.

(Slide.)

The third concern obviously is vector insertional mutagenesis and this is obviously related to all the things you have been hearing about this morning. And one of our approaches to this was we are using superficial muscles that we could easily observe for any physical change at the injection site and also each of our patients will be followed lifetime in the local hemophilia treatment center as well for any long-term complications.

(Slide.)

So what do we conclude from this early study? That the intramuscular injection of an AAV vector encoding for Factor IX in eight human subjects has been tolerated and there has been no significant systemic or local toxicities. Gene transfer and/or Factor IX expression has been demonstrated in all eight subjects. The vector shedding data did not reveal vector sequences in the semen at all time points tested. In addition the vector shedding data are consistent with the earlier animal data which demonstrated low risk of horizontal and germ line transmission.

No inhibitors to Factor IX have been detected by either the Bethesda assay or Western blot and the H&E staining of skeletal muscle shows an absence of inflammation in all the muscles studied.

(Slide.)

Now I just in a couple of minutes would like to make a comment about another trial under consideration. This is a Phase I trial of AAV mediated liver directed gene therapy for hemophilia B. The same people would be involved.

(Slide.)

This study also is -- it is an image in many ways of our muscle study but it also comes from the years of preclinical studies that have gone on in AAV Factor IX therapy in mice and dogs. Recombinant AAV mediated hepatic gene therapy is efficacious in small and large animal models of hemophilia B. Lots of data for that. And also there is no direct evidence of any related toxicity in mice, rats or dogs at doses that are five to 100-fold greater than the proposed starting dose in this particular trial.

(Slide.)

The question that often comes up, why muscle and why liver for doing muscle, but there -- I think there is -- these are two different complementary approaches. This issue, in fact, was discussed at the RAC committee last year. There is a lot of unresolved issues which will need to be defined.

What is the interval required for retreatment in muscle versus liver if

that is even possible?

And currently it seems that the vectors that are used in muscle require a much higher dosage compared with liver so there seems to be based on ongoing animal studies a dose advantage of using the liver as the target organ. And one could approach it as it might be possible to achieve therapeutic levels with the liver versus -- excuse me. Pure levels versus the therapeutic levels in muscle.

And the incidence of the formation of inhibitors is significant. There is some preliminary data in at least the null dog Factor IX model that the incidence of inhibitors might be greater in muscle -- following muscle injection and when it is directed towards the liver.

And it is for these reasons as well as the fact that the muscle probably is unlikely to work for hemophilia A that an approach -- we are looking at the possibility of a Phase I study in liver.

(Slide.)

The specific aims of this study would be to test this hypothesis that AAV mediated liver directed gene transfer is safe. I have characterized again the immune responses to the product and to the vector. Again determine whether germ line transmission of occurs from following hepatic administration. And obviously to determine if this approach will result in an improvement in the clinical course of hemophilia B.

(Slide.)

The study design will be an open labeled, dose-escalation safety trial, administered by infusion into the hepatic artery from the catheterizing up from the femoral artery. Again three subjects will be enrolled in each of the three treatment groups, low, medium and high. The low doses will be projected to reach levels of one to two percent based on the earlier mice and dog data.

This protocol has been approved by local IRBs and IBCs. It has been reviewed by the OBA and currently is being reviewed by the FDA. And this factor preparation would be made at Avigen as before.

I think because of time I cannot get into the specific entrance and inclusion and exclusion criteria but it may come up in the discussion.

DR. JOHNSON: Thank you, Bert.

Questions?

Phyllis?

DR. GARDNER: In the preclinical animal models when you administered -- I have two questions. When you administered AAV to the muscle in the clinic you see some serological -- I mean, you see some evidence in the serum for a day or so. In the preclinical animal models did you see any evidence that you got it into other organs, including the liver when you administered it by -- to the muscle?

DR. GLADER: Mark, do you have the biosafety distribution data on that?

DR. KAY: I do not know if Roland or Linda has that.

DR. GLADER: Roland might have that.

DR. KAY: I mean, there is some PCR positive signals in other tissues but I do not believe there is any double stranded DNA forms.

Do you want to say something, Roland?

DR. GARDNER: My second question is when you look at your patient population, which is to a large degree hepatitis C positive, some hepatitis B, how does that make you feel in terms of hepatic transfer?

And following up, whether it is vector related or not, you are going to have incidence of hepatocellular carcinoma.

DR. GLADER: We are going to have an instance of hepatocellular carcinoma. I think that is part of this discussion here. I do not know if it is simple. I think we -- it is the whole issue of we spent a lot of time with our hepatologists talking about this and obviously from the things you have heard this morning, as well as the fact that if you were to do this study in adult hemophilia patients, they are hepatitis C positive. I do not know what the number is, 80 percent, somewhere up there.

And the risk of hepatocellular cirrhosis and hepatocellular carcinoma is there and the advice we have gotten -- unfortunately, one of the hepatologists we wanted to have here to address this issue is still up in Boston as I understand. But the advice we had is that we are requiring liver biopsies in these -- in patients in the liver study and the -- and people who have a grade two on a zero to four scale biopsy prior -- at the -- within two years of starting this study will -- would be eligible.

And our guidance and advice for this has been, you know, excluding anything else you have heard this morning, is that this will not increase the risk of accelerating any process because these people are very, very low risk for going on to develop cirrhosis or hepatocellular carcinoma in a period of ten years. It has been given a lot of thought.

DR. JOHNSON: Mark?

DR. HALSEY: Peter Halsey, Aventis Behring. Could you comment on the stability of expression? When did you measure 1.6 and how much was it at the end of the observation period?

DR. GLADER: The one person who had a significant clinical level, I think the 1.6 was measured earlier on but even now I think it is a little over a year out and it is still over one percent.

DR. JOHNSON: Roland?

DR. DAWES: What would the dangers be in increasing the intramuscular dose?

DR. GLADER: Again there are preliminary -- Roland, you may want to comment on this. There are data that the concentration of vector per site may have something to do with inhibitor formation, which could -- you know, if you could give an increase dose or have an increased concentration, obviously it would make a lot of things easier but I -- Roland, Dr. Herzog?

DR. HERZOG: Yes. We have looked at that in the Chapel Hill hemophilia B dog model, which is a missense mutation model. These dogs have a missense mutation but they do not have any circulating Factor IX. So they have -- so they make the protein but it probably is not stable. It is either not secreted or it is degraded intracellularly.

And in these dogs we have done a dose escalation study and we were able to get long-term expression in all of the animals that were injected up to a certain vector dose. But when we increased the dose per site from $2X10^{12}$ vector genomes per site up to over $10^{13}$, which was about a six-fold increase, then we found formation of an inhibitory antibody that lasted for about a year.

So looking through all the data and having injected additional animals, it really looks like that you can actually get an inhibitor if you overdose per site. And talking to our immunology collaborators that seems to make sense from vaccination type studies that you would have to be worried about how much antigen you put into one intramuscular site.

So while -- at the same time this should also be influenced by the underlying mutation so it is very well possible that this would not have been a problem in these patients that have no history of preexisting inhibitor formation. And a lot of these patients also have actually circulating but nonfunctional Factor IX.

So they might be from an immunological point of view even more protected just to be on the safe side where we did not want to dose, you know, what gave us a problem in this particular animal.

DR. JOHNSON: A question at the table?

Jay?

DR. SIEGEL: Yes. I think you mentioned in your last slide that the proposed hepatic study had IRB approval. I was just wondering how long ago that happened and did they have access? Well, just because a lot of information is developing recently, was there discussion of consent and tumor risk in the animal data?

DR. GLADER: It has been back to the IRB several times and it is in the process right now in light of new information.

DR. JOHNSON: Mark or Terry?

DR. FLOTTE: I had another question that might relate to the rationale for the hepatic delivery. It seemed to me in that one slide we saw of immunohistochemistry from the skeletal muscle from the treated patients that a lot of the signal was in the extracellular matrix in the paramecium. It seemed a little bit reminiscent to me of your earlier mouse studies where it seemed that there was an advantage of hepatic delivery over muscle delivery in mice because of that problem.

DR. KAY: Definitely there is a 50-fold dose advantage at least using the best muscle vector versus the best liver vector and the issues around that are unclear. I mean, there are still issues about muscle vector, about using muscle specific promoters, other types of serotypes, et cetera, that may actually allow one to increase the injection dose per site without getting inhibitors at least in the dogs.

I wanted to make a comment about the HCV issue. The hepatitis C virus is a cytoplasmic virus and this is -- AAV is a nuclear virus and we have talked to a number of HCV virologists, including Frank Shizari, Margaret Cozell, who is at Harvard, who was supposed to be here today and unfortunately could not make it, and from the best of their knowledge there is no reason to think that there would be an interaction between the two viruses.

One of the things we worry about and we have looked for this is cytokine stimulation with AAV thinking that that may exacerbate the HCV and from what I understand with HBV that certain cytokines actually decrease the hepatitis B titers.

So there is really no reason to think about the -- to think that there is going to be an interaction that is going to be problematic in that regard.

The issue that Bert brought up, I think, is an important point and when we have talked to our hepatologists, many of them said we could probably go to stage 3 patients but to be safe we decided that we only want to go to stage 2 or less because again the risk of them developing HCC and severe cirrhosis over a period of ten or more years is extremely low.

DR. JOHNSON: Any other questions or issues?

DR. MICKELSON: I just have one.

DR. JOHNSON: Claudia?

DR. MICKELSON: I just had a fairly naive question. Some of the

patients were HIV positive which might affect the immune system. Do you expect any kind of issues? If you are looking at animal data here now where there might be some impact of the underlying disease on immune function and tumor development, are people looking at the HIV infected patients quite closely? I would hope that --

DR. GLADER: We have not excluded them as patients recognizing they may not mount an immune response to a lot of different things and they are --

DR. MICKELSON: I was only concerned about an increase of some -- there might be some risk of them developing a tumor.

DR. GLADER: More prone to developing a malignancy.

DR. MICKELSON: Yes, that was all.

DR. GLADER: That is a concern.

DR. MICKELSON: The other thing, I might have misread the slide because I must say sometimes I have had a hard time seeing, were there some patients that were negative by PCR but positive by histochemistry? There was one patient on the slide there. I was just wondering if that was the case, how was --

DR. GLADER: Yes. I think the -- my colleagues in Philadelphia who are doing this -- as you may be doing a cut -- you are making a different cut of where you do your histochemistry from your PCR and so it is conceivable you can miss it and that is why three different things were done.

DR. KAY: Can I make another point about that? There is two patients where there has actually been a Southern blot done and you can use the CMV promoter as a probe which will not be present in human muscle tissue and if you get a good biopsy site you can demonstrate one to seven copies of the AAV genome, double stranded genome. So there is clearly evidence for double stranded AAV formation in some of the biopsies.

DR. SAMULSKI: Can someone speak to the doses that have been used in the patients compared to the animals, whether it is translating?

DR. KAY: Does Roland want to comment? In the muscle trial, the doses?

DR. SAMULSKI: Yes.

DR. KAY: The dose in the muscle trial was predicted based on the dog studies not to lead to any serum or plasma Factor IX detection and actually we did not expect that we would see definitive low levels of Factor IX until we got to one -- let's see. It was -- the highest dose. When we thought we saw some evidence of gene expression and because of all the issues that had arisen with safety, we decided to back off from the dose escalation. And instead of incrementing by one log unit, we went up only a half a log unit into the mid dose.

DR. SAMULSKI: Is that correlating with increase?

DR. KAY: Well, at this point, no. I mean, the N is small. Between the low dose and the mid dose there really has not been any difference but you have to realize that this was done with different lots of vector and there can be a two or three-fold difference in vector titering and now you are only going up half a log so the question is, is it really that -- is it really an increased dose or not.

DR. JOHNSON: One last question.

DR. FRIEDMANN: A quick question. In your tattoo marking do you mark simply the site of the injection or do you, in fact, put the India Ink in with the vector? Do you mark internally the site of the injection?

DR. GLADER: The area is marked beforehand and then that is where the injection is made in that area.

DR. FRIEDMANN: Is the tattoo material included with the vector per se?

DR. GLADER: No, it is not included with the vector.

DR. FRIEDMANN: Is that a good idea or a bad idea? I mean, if you are going to rely on Southern -- on molecular --

DR. GLADER: That is a good question. The fact that we seem to be getting expression in all the biopsies to date but, you know, it is a very good question because it could be a hit or miss thing.

DR. JOHNSON: Okay. We are going to take a break until 4:00 o'clock. Please fill out the yellow evaluation forms that are in your packet. Also sign up for airport transportation. Be back at 4:00 o'clock to do the roundtable.

(Whereupon, at 3:42 p.m., a break was taken.)

ROUNDTABLE DISCUSSION

DR. JOHNSON: Okay. We are pressed for time here. We have got a large number of questions for panel discussion 2. Claudia is going to lead this discussion in a very precise and focused way so that I can get home basically. I am flying out.

We are going to finish on time here come hell or high water or snow.

So I am going to turn it over to her to let her lead the discussion and, please, again, as you did in the first panel discussion, let's try and focus on the questions as much as we possibly can to get the answers that will help the OBA.

DR. MICKELSON: Most of you should have your panel -- your questions in front of you for panel discussion 2. And, although they are not up on the screen, essentially they have to deal with not that there is information about tumors in laboratory mice. The FDA placed all AAV clinical trials on hold. Studies were permitted to resume with additional data indicated the findings appeared to be isolated to the experiments with AAV in the MPS VII knockout mice and re-review of the clinical experience with AAV products indicated an acceptable safety profile.

So some of the questions, at least the first one, is should clinical investigations with AAV containing products be halted until additional data addressing the oncogenic potential are available and, if so, which data should be obtained prior to the resumption of clinical studies?

Hopefully, we can hear enough intelligent opinions on this that we can certainly give some direction or at least make a -- does anyone feel that the data that has been discussed and presented today bodes enough that we should be suggesting that clinical investigations with AAV vector backbones be halted until additional data comes up?

DR. GARDNER: Well, I will jump in because I always like discussion. It seems to me that based on what -- the carcinogenicity model that was presented that that particular transgene construct AAV with the particular transgene and particular promoter has to be reassessed repeating the preclinical animal model to see that it was not some contaminant of the vector, as well as looking at some of the parameters.

Having said that, I would also wonder -- and I asked Bert Glader, my colleague here, about the setting -- because there is enough of a red flag, should that be repeated. I would wonder in the hepatic administration of an AAV transgene vector in the setting of something like hep C, whether or not you might need more studies.

So I asked him if whether you needed to do animal -- if there was an

animal model of hep C because it would be very nice -- it is going to be very -- if there is not is what I heard but the danger to me is that you are -- you know, as you follow these patients out, they are going to get hepatocellular carcinoma and nobody is going to know whether it was transgene. I mean, you are going to have to make a guess whether or not there was any effect of the transgene and that is just something that is going to confound things for all of AAV if you are not careful.

DR. MICKELSON: Any other comments?

Yes, Mark?

DR. KAY: Actually that is another area we work on are animal models for hepatitis C and the only animal models to date are chimpanzees. We have been making xenografts of human hepatocytes in mice and have demonstrated successful hepatitis B and D infection but we have not yet succeeded with hepatitis C, and we and a number of other groups are trying to do this.

With that said, we have consulted with some of the world leaders in this field and nobody feels that the current animal models for hepatitis would be valuable for these kinds of studies.

DR. MICKELSON: Dr. Zeitlin?

DR. ZEITLIN: I think that the issue of all clinical trials on hold is an important one and I would propose that each trial be looked at specifically for the risk factors related to the disease, the age, maybe the gender and the method of application. So how far is this vector going to penetrate? For example, in our inhalational trials we at least have preliminary evidence that we are not getting dissemination, making hepatic tumors much less likely.

So I would sort of ask that we look at it on a more individual basis.

DR. MICKELSON: Dr. Gordon?

DR. GORDON: I just think that before a decision like that is made, you know, I think it is useful to think clearly about what kind of information is actually before you and how to react to it. And to segregate in your own minds what is speculation about a causative effect versus what is established as a causative effect, take for example the issue of underlying hepatitis. It is certainly true that those people have some risk of getting carcinoma from hepatitis C but for all we know right now AAV may protect those people from getting it. We do not have any evidence whatever that it would aggravate it and all of this is actually speculation.

So in my own view until this single incident under these specialized circumstances is validated, I do not think it is justified to hold up clinical studies of the vector.

DR. CRAWFORD: I was just going to make one comment about the difficulty of hepatitis C models. There was a report out of Tulane some years ago for an in vitro cell culture model which has not been replicated. The most recent is the Replicon model where it is an incomplete hepatitis C cell culture model, which we actually have no operative in Florida, and it is conceivable that you could do some kind of Ames assay or at least look at the effect of AAV on replication of the hepatitis C genome.

But I think the ability to extrapolate that sort of ex vivo work to the human condition is still a very long ways away so I do not think you are going to get experimental data from hepatitis C type models that is going to help directly with the human condition in the near future.

DR. MICKELSON: Any other comments?

DR. AGUILAR-CORDOVA: Yes, I have a comment.

DR. MICKELSON: Dr. Aguilar?

DR. AGUILAR-CORDOVA: So with regard to also the data that is presented, I think that it is also interesting to note that Mark mentioned that there would be up to two logs difference between the various lots of vector that they are producing. I also saw a significant variability in the infectious units or infectious units versus the particles. Given that some of these tumors might not have even contained the sequence that we are talking about, were all these materials tested for any contaminating product?

And so without really standardized methods or well-characterized vector, it is difficult to assess the causing agent. And this applies for any toxicology study if it is going to be taken as a real toxicology study.

DR. GARDNER: Even if it helps prevent the appearance of hepatocellular carcinoma, one can anticipate that there will be a controversy when that arises so to have as much data available is extremely important even -- because the public outcry is going to be very large. They are not going to understand the nuances I can assure you about this. It seems to me that one has to plan for that event.

DR. MICKELSON: Dr. Flotte?

DR. FLOTTE: Just again a comment not so much about the hep C consideration but I think again in terms of considering the data as a whole, we have a virus that probably 80 percent of us in this room have been exposed to in one form or another and have never been associated with the formation of a tumor even though we know that some of those viruses do not have functional rep.

We have <u>in vivo</u> experience from a wide range of laboratories and other models that do not show an incidence of carcinogenesis that is consistent with the current experiment under discussion so clearly there is something different in that. We show that statistically in our case but it is certainly different by inspection with others.

And on the other -- and we have a system that generally has no dose limiting toxicity. It is the -- it is one of the safest biological agents that has ever reached this stage of development.

On the flip side of it we have an agent which many groups, including Dr. Sands, have shown after a single administration holds tremendous promise for correction of long-term genetic metabolic disorders. And I think first of all my own opinion, admittedly biased, is that it would not be fair to the disease communities to halt trials certainly altogether. But I think also one has to be very judicious in terms of even restricting the trials based on incomplete datasets or data derived from studies that were done for other purposes.

You know, I think understandably so there is a tendency towards taking the most conservative approach but I think taking the most conservative approach does not -- should not always entail sort of worst case scenario thinking and so I just want to lay that before the committee.

I think if you look at the data in the aggregate it certainly would support compared to other agents that have progressed through clinical trials, it would support continuing with attention to this particular complication.

DR. Knazek: I just want to mention that there are quite a few hepatitis C positive chimps that are available for study at the primate centers around the country and that we could certainly help with that.

DR. JOHNSON: Yes, that is --

DR. Knazek: Lots of chimps.

DR. JOHNSON: It is a lot harder to do experiments in chimps than it is humans.

DR. Knazek: No, I know.

DR. JOHNSON: And to our knowledge they do not develop hepatocellular carcinoma after hepatitis C.

DR. KAY: And the problem is doing enough animals to get a statistically significant dataset. I mean, one thing I would say is I think before we make too many decisions about HCV issues, it is unfortunate that there is no hepatologists or HCV virologists on the panel and we actually had invited one who had agreed to come but again because of the weather issue -- but it is something that we have really taken extremely seriously and talked to a lot of people about.

One thing I would like to point out is that there were some issues raised about the ethical nature of doing liver biopsies on these patients before treatment and I just want to point out that at most centers now it is recommended that before patients go on antiviral therapies that they do get a liver biopsy to stage their hepatitis C.

DR. BREAKEFIELD: I ultimately think that this decision -- this very pithy decision resides with the FDA and I think people have different views about how they would handle it if it was their choice. I would say that I am sort of more of the -- just personally more think that in each trial the route of administration should be taken into account.

But given this data I am a little uncomfortable, you know, going directly to the liver at this time without more information myself, personally, I would say. And I think the FDA has to take -- you know, they have their own group of people and they have to decide for themselves whether in hemophilia, which is not life-threatening, are they concerned enough that they would like to see a little more data before they put this into these patients.

DR. MICKELSON: Other questions?

DR. SAMULSKI: Can I make a comment? You know, first of all, I think Mark Sands should be applauded for bringing this out to the open. Technically if he would have taken the approach of trying to reproduce this, no one would have known anything for two more years. And we would not have even addressed any of these points. And he may either have reproduced it or not reproduced it and it has gone by the wayside.

But be that what it may, the community has been presented with a scenario that says we have an unknown here and how do we go forward and take into account the patients and take into account the appropriate scientific approach.

And from my perspective I have seen no data today that has argued that the viral vector is an insertional mutagen. Even from the most simplistic approach that it should be present in all the tumors has not been substantiated.

Now granted we need to do experiments to see it technically. We saw data for the first time. But if you are going to use this type of approach to stop the field completely, you need to ask how many people are going to generate data that may not be as good as Mark or as cautious as Mark that will bring to this committee the potential of stopping everything again and what are we ultimately going to end up doing resolving scientific problems at everybody's lab?

Are going to take a logical approach at how to move forward?

So I think I would like to make it clear that my assessment of this is that I have not been convinced one way or another that there is any risk factor or that AAV is not responsible and that data has to be generated but at the same time I do not

see how this body should be making statements about policy for whether clinical trials should go forward.

I think the FDA should chime in and give us some guidance here because we are clearly out of our league and we are making some suggestions that probably are not appropriate.

DR. SIEGEL: Well, I guess it is worth nothing that the question does not ask specifically whether the FDA should put these trials on hold. It asks whether this research should be halted because, in part, in fact, this committee has not been briefed or educated on what the FDA regulations are, what are the rules, how we deal with incomplete information. And, in part, to reflect the fact that this is a bigger picture.

The question as to whether to conduct these trials or to wait until there is more safety data available is a question that needs to be addressed by investigators, by IRBs, by granting authorities and by regulatory authorities.

So I did not really mean to focus it or we did not mean to focus it too much specifically on what an FDA decision should be. Nonetheless, the FDA will -- you know, is continually making a decision. At one point in time we made a decision to stop everything but we got more information and as we got more information we made a decision to allow, as we always do, on a case by case basis but we will constantly reassess that.

Any decision this committee would or could make would perforce be made at one point in time. And although most of the experiments we are talking about are going to take a couple of years or longer, there will be more information obtained. There has been on a weekly or every few weeks basis over the last two or three months and there will be more information obtained in the near future so it is very hard, I think.

I think I am not sure I fully understand your question but I think it is -- it would be difficult for this committee to make a judgment on this field of science of do not do the research or do the research but rather we are hoping, though, to elicit what are the considerations and based on where we are now what is or is not an appropriate way to proceed.

DR. JOHNSON: I think the role of this committee probably is better viewed as scientific advice rather than regulatory advice.

DR. MICKELSON: Absolutely.

DR. JOHNSON: I mean, I think there are plenty of regulatory issues that we have not even begun to address today and rather we have been trying to judge the body of science that points out -- I mean, this is the first time I actually have seen the data or heard, you know, anecdotal reports and so on and so forth.

So I think, you know, it takes time to digest this and there is always a reasonable middle ground when you are faced with situations like this, I think, and I think you just -- you know, basically stated that one moves ahead with caution constantly evaluating data and constantly evaluating the situation and at the same time move ahead with the science, and go ahead and gain additional new knowledge that will help down the road.

So I agree with what Jude said. I have not seen anything today that made me believe that there is -- that AAV is an insertional mutagen. It does not mean it is not but today I do not think that reasonable science would say that it is.

So, therefore, what we are left with is the fact that there are a nice dataset that needs to be addressed, the experiments need to be repeated, maybe

additional controls need to be done. That is going to take time. At the same time it seems to me that this body should not make any statements about hold on clinical trials, et cetera.

DR. MICKELSON: Eric?

DR. JUENGST: Yes. As kind of the layperson on the committee I wanted to echo what Dr. Samulski was saying. First of all, in congratulating you for bringing this to the public. That phase of the process, I think, is a really healthy move and something we should encourage even on, you know, other kinds of reports that might be dismissed even more easily than this one.

But my sense sitting here listening, as best I could, was that the answer to this question should clinical investigations be put on hold is no.

There is an answer, though, to the second part. There is some data that still needs to be gathered and that is the repetition of the experiment, et cetera.

DR. MICKELSON: Basically it is trying to assess our comfort level with what we heard today. I would certainly agree with Dr. Gordon's comment. I would think -- I do not think that we saw data today that would be enough to ask or to even feel that we should be looking to have the clinical investigations with AAV products halted

Of course everybody is interested in more data and repetition of the study and looking to see if this was something specific to that particular combination of transgene and mouse model so that people can put the appropriate things in place.

My only concern from what we have heard today is something we will discuss later is what is it that we are going to ask investigators to tell their patients now about this and what are we going to ask them to look for in their patients?

DR. JOHNSON: So is that question number three?

DR. MICKELSON: I think that is, yes.

DR. JOHNSON: Okay.

DR. MICKELSON: So is there anyone who feels that we should be halting the clinical trials? Speak now because we are moving on. I did not think so in this group. See me later if you feel that way.

(Laughter.)

DR. SLY: Could I ask just one clarification?

DR. MICKELSON: Certainly.

DR. SLY: Are there any clinical trials that involve this particular promoter CMV construct?

DR. MICKELSON: Other than at the moment, no. No, no clinical trials.

DR. SLY: Okay.

DR. MUZYCZKA: There is some plan that involves that CMV promoter, yes.

DR. MICKELSON: Yes.

DR. SLY: This exact construct?

DR. MUZYCZKA: No, not the beta glucuronidase construct.

DR. SLY: No, I mean basically CMV.

DR. MUZYCZKA: Beta actin, yes.

DR. GARDNER: I suppose that I would say that I do not think this is -- I mean based on everything we saw today if I had to place a bet that it was AAV insertional mutagenesis, I certainly would not halt airway trials. I would not halt muscle trials. But I started to get more nervous as we approached the precise model

where this was seen and so as you replicate the route, you replicate the vector, you replicate the promoter, then you replicate the transgene, the closer that you get to what caused it, I would get more uncomfortable.

So I am not quite ready to say halt. I mean I would not halt some particular human trials that might have elements of this. And one element is direct hepatic administration and another would be with this same promoter, et cetera.

So I just wanted to throw that in.

The one thing I also wanted to bring up was I think that the experiment -- everybody says, well, you have got to repeat the experiment but it is a two year experiment and it is probably a costly experiment. And, indeed, if you wanted to do it and not wait two years to see if this is repeated but test some of the other factors such as strain, specificity, transgene specificity, promoter specificity, it is a pretty big trial on mice. I mean, you are going to have to look at different lysosomal enzymes. You are going to have to look at different promoters. You are going to have look at different routes of administration, et cetera.

Who pays for that?

DR. MICKELSON: NIH.

DR. JOHNSON: Where is Kathy? She is disappeared. Again I think we can deal with that in the working subgroup. I do not think we are going to answer that today. There are mechanisms by which it can be funded. I am certain of that.

DR. SANDS: If I can just make a quick comment, and it is really to just highlight what Jude said. I have had the luxury or, if you will, the headache of thinking about this longer than anybody else in the room. And having had more data generated, seeing Terry's work, talking to Mark Kay, Kathy High, there is really no answer here. We have no mechanism. Nothing right now points to AAV. On the other hand, nothing points to MPS VII yet either.

There is really -- we have no information and none of these studies, our study, Terry's, nobody's study really was designed to answer these kinds of questions. So to try to make a decision, I think, is extraordinarily difficult and I do not have an answer and I am not willing to voice an opinion on whether trials should be stopped or not. That is not my place.

But I also think we do not have enough information to really make, you know, the best educated decision. I think the best thing we could probably come out of this meeting with is a plan on how to set up or perhaps set up a toxicology study and really get to the route of the problem.

Now in the interim, you know, I do not envy the FDA at having to wrestle with the issue of these clinical trials because there is not enough information there.

DR. SIEGEL: Would you concur -- I guess there were opinions expressed by Drs. Breakefield, Gardner, Zeitlin and perhaps others that not -- in this state of uncertainty there is probably more concern about administering vector to the liver than say local administration. Would that be your perception as well?

DR. SANDS: That is my perception only because that is where we saw the tumors and when we give a high dose injection of virus most of the virus goes to the liver. Now exactly how much gene transfer are you getting with an aerosolized delivery of virus, is it comparable to what you are getting with a bolus injection in the liver? I have no idea. I do not have even know how to measure that.

So I think the fact that we saw hepatocellular carcinomas, you know, makes us all think about the liver but again there is just not enough information there.

I do not know.

DR. JOHNSON: Again I think we have to make sure that we are keeping our eye on the data and letting the data drive decisions and I am not sure that even though you are right -- I mean, we do not have data that would make anybody comfortable 100 percent one way or the other. I think there is enough information that we can say that this group should try to help direct the science. And that seems to be me to be the best thing that we can do.

Don't forget, also, that you had tumors outside the liver as well, a uterine tumor and a tumor at the base of the brain. So I think that, you know, it is not fair just to say the liver because you did have tumors at other places. So I think it is dangerous to start going down that pathway to single out a single organ and say, you know, we cannot do it in this organ because vector will get to the liver no matter where you put it.

DR. GARDNER: You could say local versus systemic administration and you can also look at the question of on local administration -- for example, the airway epithelial turnover and slough very quickly so your dose is going to be a different thing. In muscle you have yet another issue.

When you have to make a guess at every one of these -- and I were the FDA I would not halt airway trials -- believe me, this is not a personal thing. I think there is a lot more safety data on the airway. In terms of the hepatic it was not just that but it was a high dose systemic administration.

DR. SIEGEL: Could it not be though that, in fact, the exposure to a given cell is higher in certain local administrations than in systemic and that maybe the risks are higher?

DR. AGUILAR-CORDOVA: Yes. So I think I want to echo a little bit of what Jude said and that is that there is no data to support the conclusion at this point from what I have seen. I cannot come to the conclusion that the vector was a causative agent in any of this and I imagine that if we were to receive a manuscript that said that this caused tumors, AAV caused tumors, it would be rejected as a conclusion.

And so I see that it would be difficult to go as a regulatory agency and say we are going to stop any trials based on scanty data which cannot be used for a definitive conclusion in a scientific manuscript.

DR. SIEGEL: Let me just assure you that would not be difficult. That is not a problem. We do not require an extremely high standard of proof that a treatment is harmful in an experimental chimp before we stop the experiment.

DR. AGUILAR-CORDOVA: But at the same time I would imagine that there would be some semblance needed to have a good indication or strong indication prior to just using basically a phenomenology like it was said that was not designed as a toxicology study and so at the very least one would need that.

DR. MICKELSON: Ted?

DR. FRIEDMANN: I do not know quite how to say this so I will say it carefully. First of all, I think that the science we heard today and the process of all this procedure has been wonderful. I think that this is exactly what the scientists and the clinical investigators and the FDA and the OBA should be doing. So I think that this has been a very, very sort of constructive and optimistic and useful exercise, and I think it is all going in the right direction.

I think we have gotten a little bit too tied up in the discussion today with mechanism that we all admit we do not know and we all expect that if we see

tumors develop in these gene transfer kinds of procedures that they must have to do with insertional mutagenesis and the data today convinces all of the people who have looked carefully that there is no indication that convinces anyone that there is insertional mutagenesis involved in this phenomenon.

But we did hear today people -- a number of people say that it is looking like that something in this material, in this product, as partially characterized as it is, the fact that it is not fully GMP characterized material, something in this material may have had something to do with the development of tumors in this case.

And, therefore, rather than worry about explaining it on the basis of insertional mutagenesis, I think what we should be asking is in this material is there something that is related to the development of tumors?

And I think a manuscript sent to a journal saying that in this system AAV has done something would probably be just as likely to be rejected as a manuscript saying that AAV in this material caused a tumor because we do not know what the mixture of stuff is in this preparation.

So I think what I am saying is that I have a slight feeling of discomfort of mechanism. We do not know what the mechanism is of this event. Whether it is related to AAV or not we do not know and that is why, of course, all of us are so anxious to see a repeat of the experiment and why it is so terribly difficult to know what to do in the meanwhile.

But I think to say that we have not seen proof of insertional mutagenesis and, therefore, we should not be worried about the mechanism, I think that probably that is a bit of a mistake and we should be very careful to say that our first job now is to know if we can repeat the experiment, repeat the phenomenon, and if we can, in fact, repeat it with material that we have fully characterized and we know is, in fact, AAV related rather than related to something else in the preparation.

So I agree completely with Jude's comment and all the other comments but let's not get too tied up with mechanism. Let's not get too tied up with trying to attribute it to insertional mutagenesis. We have heard this material described earlier today as a carcinogenic product possibly and if you just call it that rather than AAV then I think it may shape the decision.

DR. MUZYCZKA: Let me just make one quick comment because I have to leave. I want -- I agree with Jude and with what Estuardo said earlier. On the evidence we have heard today, and I would take issue with you, Ted, the evidence we have heard today basically allows us to conclude that there may be a problem in the MPS VII model and that is it.

On the issue of the vector we heard plenty of evidence that said regardless of how you felt -- clearly there is no insertional mutagenesis involved here or very likely there is not. We also heard a lot of evidence that says there is nothing else in that particular stock that is involved here because we had a large number of experiments that are -- a number of experiments equal to the ones we heard on the tumor side with essentially the same type of material, the same preparation material and there were no tumors there.

So the preponderance of evidence, as I see it, says there is something unique about this particular model when you correct it that creates a tumor. Whether it is the IGF receptor issue or something else I am not sure. That is where the evidence is pointing me.

That kind of evidence says we should not stop trials. We should do a fairly conventional and what will have to be commonly conventional toxicology,

oncology tox study for viral vectors. Since we have never done such a thing before, we will have to design it. But I do not think we should be stopping trials and I would say no to all these questions that you have here. They all assume that we have shown that we are going to get tumors from this thing and we have not shown that at all.

On that, I have got to go.

DR. MICKELSON: Phil?

DR. NOGUCHI: I would just like to reflect a little bit on what this public discussion is all about. There are several of us in the audience who actually participated in the first gene therapy meeting at the Cystic Fibrosis Foundation a number of years ago where the issues were perhaps not quite as dramatic as this was but the type of interaction was precisely like this. This is precisely the kind of interaction and sharing of data, spurring each other on, and not being complacent about data that I think really leads us on.

Ironically if you look closely at the clinical studies that were talked about in depth today, Dr. Zeitlin in particular, that all came from that cystic fibrosis meeting, which was all about adenoviruses. All that discussion really led to the conclusion that if you are going to use adenovirus in cystic fibrosis it had to be done in a special way and many people just said, "I have seen enough. Let's move on."

That is where AAV discussed. That is where retrovirus were discussed. That is where all the plasmids, the gutted adeno, and I would say that what we hope -- one of the purposes that we hope that this discussion really shows you is you cannot be complacent in science.

Terry, you made the statement that this is the safest vector so far. We would not look at it quite that way. What we would say is that it has many properties that is compelling for clinical trials but all biologics have consequences. Some of them minor, some of them major.

We can deal with them but we need to deal with them together because I have already heard today about seven different separate collaborations which will lead in different directions, some of which will address the tumorigenicity issue. Some of which really should start addressing how do you control the expression if only a few cells get transduced when it is kind of evident here that over production at very high levels may in some cases not be the best idea.

That is a factor for hemophilia where you want enough but not too much and where the current clinical data -- why don't you want to just enhance the muscle? Well, that is being based on animal data that is being generated right now. What we surely do not want are inhibitors because then you have to use nonhuman products to treat hemophilia. That opens a whole other bag.

So at least for at least some parts of this one of the biggest lessons here is, you know, biology is exciting but it is also very tricky. The evidence that Mark Sands -- and I will echo everybody for him bringing that to both the NIH and FDA's attention -- absolutely needed to be discussed and it needed to be shared, and we need to get more good minds on this, and we need to get more minds that may not even be interested in tumors or in GUSB or in MPS VII but are interested in perhaps the mechanics of delivery.

That is what these CF Foundation meetings really did. It brought everybody together who may not have had a direct interest in CF or in the gene or any of those things and, in fact, you really saw Mark's interest was in curing mice. Well, that does not mean his data is suspect at all. This is the very essence of basic science and I challenge the basic scientists to just look at it that way.

Go where the data lead you. Yes, we want to have some input as well on what do we do with clinical trials but, you know, I mean it is a two way street. Do not be afraid to share this information with each other and move from there.

DR. MICKELSON: Louise?

DR. MARKERT: My opinion is that one should go ahead with AAV vectors even in the liver. I think I understood this right in terms of Mark's studies that the tumors were just in the group of mice that were injected neonatally and those neonatal mice have -- I mean, those are totally weird. You know, they are different than mice that are a week or two old or three weeks old. They are totally different than neonatal humans. Neonatal humans are much more advanced immunologically. And it may be just something related to that very immature state that we would never see or, you know, that certainly is different than the humans and later.

But I think we need to see a lot more data certainly to convince me that there is something that can be applied to everybody from this particular situation.

DR. MICKELSON: I think I would like to refocus us back on to the questions and since there are no further comments, no one standing up for question one, I think that in general most of the comments have said that people do not want to halt any -- to halt clinical trials but that certainly there is some data -- and experiments that we would like to see done, of course, continuing studies.

But the second question deals with are there some limits on -- in terms of clinical trials in terms of patient populations that AAV -- we might consider being more selective in terms of AAV patient populations. Considerations on use only in serious or life-threatening diseases, age, liver function, the presence of pre-existing viral infections.

And that whether anyone feels that these kinds of issues should be taken into account for patient population selection where AAV would be the delivery vector. Not even routes of administration but just AAV in general.

Nick, had a fairly succinct statement known to all of all of the things in question two.

Does anyone want to comment?

Terry?

DR. FLOTTE: I already gave my generalized impassioned plea before but I want to speak particularly to the age related issue. I think something has to be distinguished here, which is that newborn mice are weird. I am glad that went on the record that there is -- you know, a newborn mouse in terms of immunologic parameters, in terms of the proliferative rate of various organs that are a special case, and that really does need to be paid attention to in the science.

But I guess part of this comes about as being a pediatrician and a pediatrician interested in taking care of patients with genetic diseases that in -- if we do want to use this vector, and I think most of us are generally using it in diseases that are life-threatening or serious right now.

On a case by case basis in some of those that only really make sense, the only good trial to do ultimately might be in a pediatric population. So I guess I would say as echoing some of the comments earlier in terms of case by case assessment, I mean we have had again a strong history of support for the AAV community from the NIDDK and from other organizations interested in genetic and metabolic disorders. And I think we might end up -- if there was a general age cut off, for instance, we might end up excluding doing the right trials for certain diseases and I will not go into too many specific examples but I think the point is it would be a

different answer in terms of the risk/benefit ratio for different particular disorders.

DR. MICKELSON: Other comments?

DR. GORDON: I completely concur with the notion of a case by case evaluation for administering this rather than a blanket judgment on the disease entity. That analysis can cut both ways. One can make the judgment that in a serious disease entity it is a greater risk to use it because the patient is more delicate. Whereas in a very mild entity it may not justify the risk. I mean, one can argue themselves in circles this way ad infinitum without resolving it so I really think that when a physician is confronted with the patient they need to look at the patient profile on a case by case basis and decide all things considered whether it is a reasonable judgment to use it.

DR. MICKELSON: Any other comments?

DR. KAY: As a medical geneticist as well who treats kids, I would totally agree with that and I guess one question I had was if you had a patient with a severe lysosomal storage disease that would be considered a lethal illness and you had good efficacy data that you could really improve the quality of life for those individuals and yet we knew that there was some probability that the treatment might lead to some tumor or some cancer, would you treat those patients. If you had a severe patient with MPS VII, would the risk/benefit ratio be in the direction of treatment even with this possible risk?

DR. SLY: Well, I think that is an interesting example because there are no alternative treatments with these very rare square diseases or diseases for which there will be no company ever developing therapy. So these patients will ultimately have to treat themselves and that is why gene therapy is appealing.

But in answer to your question I would be a little -- I would certainly be hesitant to use this particular vector and administer it intravenously for hepatic uptake. I would prefer to use enzymes. I would hope that there would be enzymes therapy that could hold them during -- for another two year trial or something but there are many other diseases where I think that does not apply.

DR. MICKELSON: Any other comments?

It just seems that most comments were case by case basis with some reference to consideration of the particular age sensitivity of the population, in particular neonates. But you are right, in most cases they are life-threatening diseases. I certainly would agree about age related issues for neonates but risk/benefit analysis is very difficult. That is why you have committees so that you get a group consensus so that a risk/benefit ratio is a mutually agreed upon discussion for clinical trials.

Are there other comments on any of the other issues embedded in question number two?

No comments about testing liver functions or existence or pre-existence?

DR. KAY: I have a comment about liver function. We discussed this with our hepatology colleagues for screening HCV infected patients and there is no good correlation between the liver function test and the severity of the disease. In fact, if a HCV positive patient has substantially elevated liver functions there is usually something else going on, especially if it is a prolonged thing and from all the clinical studies the best to my knowledge and the way I understand it, there really is not a good correlation and that is why we feel the necessity to do the liver biopsy.

DR. CRAWFORD: I would also echo that and the same could be argued for cystic fibrosis. There can be an intrinsic elevation in alkaline phosphatase

because of the disease affecting the biliary tree so that to make a blanket exclusion on the basis of liver function tests I think might be erroneous.

DR. GREENBLATT: Yes. Jay Greenblatt, the National Cancer Institute.

It takes a lot for me to get up and talk because I am kind of nervous and I do not like to do it so I kind of feel strongly about this. Let's look at what we know. We know this product which happened to contain AAV in a particular strain of mouse with a particular disease, lysosomal storage disease, which was done at a particular time with no controls which in a particular animal room at a particular institution by a particular investigator happened to result in tumors.

I just cannot see based on that that there is enough evidence to really restrict AAV research in humans to serious or life-threatening diseases.

Thank you.

DR. MICKELSON: Dr. Gardner?

DR. GARDNER: Well, I would like to just go back to the early days of cystic fibrosis when we discussed this in which adenovirus was shown to have a major inflammatory response with certain investigators under certain conditions and we went ahead with things, and I am not saying that we should not go ahead. I actually believe we should go ahead with all the trials. The only one I would examine carefully and just think about is whether direct hepatic administration is warranted with the -- I mean, with the same transgene, same product, I would certainly say I would hold. But with a different transgene maybe not. Just be aware of it. Be aware and anticipate it.

But I will tell you that if we should -- one patient get hepatocellular carcinoma, and people look back -- look at the Jesse Gelsinger case -- it is going to have a profound impact on the stock market, on funding of trials and everything else, and you have to anticipate that.

DR. MICKELSON: Not to mention the patients.

DR. GARDNER: Not to mention the patient. I agree with that but if you remember that also out of this good therapies were made -- I mean, there are multiple products being tested, multiple indications, all of -- I mean, 90 percent of those might be safe, 10 percent might not. You cannot under estimate the danger of proceeding ahead without extreme caution and informing everybody of that so that that kind of thing does not happen.

DR. MICKELSON: I just had one -- well, Dr. Flotte?

DR. FLOTTE: Yes. I just wanted to make -- you know, also being from the CF old days so to speak that we have been reminiscing about. The adenovirus inflammatory toxicity was really not an isolated finding in one lab. I mean, in multiple labs showed that it was reproducible, dose related, dose limiting toxicity. A property the vector shares with the native virus. So it kind of fit into a context and I think everybody has said who has committed on the science here that we are having trouble fitting this current finding either in the context of the vector or in the context of MPS VII. I think have some very elegant studies have shown about the models and about over expression of transgenic protein, so we are having trouble fitting this into a context. But I think it is different.

So I agree with you. I agree in the sense of the caution but I guess to me this is qualitatively different when one has an isolated finding in a particular system compared to a demonstrably reproducible vector property, and that is what we do not have here yet. So it requires further study certainly.

DR. MICKELSON: Certainly.
DR. FLOTTE: It is different.
DR. MICKELSON: But what happened when the first experiment came forward that showed Ad5 was -- had -- was inflammatory and elicited response?
DR. JOHNSON: Everybody said, "I told you so."
DR. GARDNER: You know, I am honestly saying that we should go forward but I do remember those days and that with the first time it showed or the second time I do not think everybody said, "Oh, it is totally expected." The people who were doing the trials argued to move forward that it was vector related. A lot of us did not believe that but a lot of times they said that and they said it was not -- it was mode of administration. It was mode of bronchoscopy. It was this. It was that.
I do not think that everybody jumped up and said, "This is absolutely expected." Not in those early days.
DR. KAY: (Not at microphone.) (Inaudible).
DR. GARDNER: I do not think they are exactly comparable. I am saying that should an adverse event happen down line we as a scientific community have got to have anticipated it, have instituted the studies properly to say that we not taking it for granted.
DR. GORDON: Can I just make a quick comment on that? The logic being voiced at the table is completely air tight but the problem I have with it is how -- what kind of a corner are you going to put yourself in. I can tell you that if a devastating event occurs in somebody who receives AAV, let's say they got HCC, and we decided or you decided or FDA decided no direct hepatic administration, the comment made the next day would be, "Oh, they took --" would not be "Oh, they took adequate precautions." It will be, "Didn't they realize that some of this AAV would get to the liver if they put it in muscle?"
There is no way that you are going to be able to defend yourself against that type of Monday morning quarterbacking and I think that is a lesson actually of some of the adenovirus debacles is that you cannot get out of that. So, therefore, I think you should look at the patient and say whether or not you as their physician are making the best judgment for them.
DR. SIEGEL: I think you are right. You know, I have been being second guessed for the last 20 years or so. I do not anticipate ever getting out of that. I am not uncomfortable with that but I think that Dr. Gardner is making an important point in drawing that parallel and thinking about its impact on the science because if you treat a patient who is at baseline high risk or even moderate risk for hepatocellular carcinoma, you are creating a risk that that patient will get a hepatocellular carcinoma that will, even if potentially incorrectly, be attributed to the intervention you do and that second guessing will happen.
And so that is at least something to think about in terms of thinking about what experiments to plan and how to proceed because, you know, if the patient develops hepatocellular carcinoma, even if it appears that it might well have happened without the intervention, it is likely to have a rather profound impact, not just on that trial, but on the entire field of AAV research and the entire field of gene therapy, and possibly the entire field of clinical research.
I think that is the point you were making and that is something that, you know, is not a scientific question but it is a question about how one should best proceed scientifically.
DR. _____: I would just like to point out that the indexed event

that brought everyone here is an evolution of several tumors very late in the life span of the animal in question. Given all the other caveats about the nature of the experiment that was done.

And in the course of human clinical research people with hepatitis C are at high risk over their life span for acquiring hepatocellular carcinoma. For the investigator who wants to do the study involving AAV in the liver, it is okay to say that one needs additional studies but I do not think within the time frame that people can operate that there is a paradigm to really address the question regarding the event in the patients as has transpired in the animals.

There are no markers that one can look at in a model which would identify within a matter of weeks that a tumor will materialize in a number of years. So, unfortunately, it is really a binary event. One can do more and more experiments but they are really in the nature of being able to tell somebody later how much you did, not really in the nature of identifying the risk that the patient is subject to.

The other side of that test is if you do all these things and the work does not get done, it is conceivable that AAV Factor VIII, Factor IX administration to the liver actually has a salutary effect on the course of hemophilia and that does not happen for many years while one is doing these other things which do not really identify the likelihood of the problem.

DR. MICKELSON: There is no one standing.

(Slide.)

Question three was prior to lifting the clinical hold the FDA required sponsors to modify their consent document to inform patients of the laboratory findings or experiments. From what we have discussed here, what information do participants here think should be included in consent forms? What kind of discussions would you want to have with potential participants?

Eric?

DR. JUENGST: I think all the same arguments we have been making apply here. I would say that if we are approximating the paradigm then, of course, you want to report these results to the patients in the informed consent form but the further you get away from that paradigm the less need there is to do that.

So in most studies I do not think it is a material risk of the study and there is no need to include it.

DR. MICKELSON: Okay. So you would not think that you would include some information on this the informed consent and might go ahead with AAV but only in ones that were more closely like the animal?

DR. JUENGST: Right.

DR. MICKELSON: Okay.

Dr. Zeitlin?

DR. ZEITLIN: Well, we are already in our past versions of the consent forms discussing theoretical risk, theoretical risk of cancer is in our consent forms, and I think as soon as you have a little more data that is out in the public arena about tumors that we cannot leave it out of the consent form.

Our patients are on the internet. They are reading the New York Times. They called us right away as soon as they heard about this through the newspapers. So I think we are obligated to present it in a reasonable fashion so that it does not raise undue anxiety and that is really the problem I have now is how to keep the anxiety level at a reasonable level and not have a lot of fears on the study population.

DR. MICKELSON: It is very hard to present experimental data, I think, in terms of the consent forms that I have read and that I do not know whether patients always understand that they are in an experiment. Also it is very difficult to explain to them that you do not happen to know which strain of mouse they are going to be. Are they going to be in the very sensitive end of it or are they going to be the ones -- nevertheless, how do you interpret this data to patients but I think it has to be there. I do not think you could have a clinical trial now with the information out in the public arena without some mention of it to potential participants.

The language, though, I think is very difficult to make sure that it is clear but also I do not know if the FDA would be willing to share what they actually asked for in some of the informed -- in the informed consent.

DR. WEISS: Actually I was just wondering if I could actually ask maybe somebody from -- who has been participating in the Targeted Genetics Study, they actually provided us a sample of the consent form that we looked at. But we discussed the elements that we thought would be appropriate to mention and then the process was that the company said, "Fine," and they basically made some revisions, faxed them over to us and we looked at that. We, I think, had very minor comments. I did not actually bring a copy with me but I was just wondering if somebody from just the company would be willing to just mention --

DR. JOHNSON: Tom, if you could?

DR. MICKELSON: Share the language?

DR. JOHNSON: No, give -- you can paraphrase it, Tom, which I am sure you can.

DR. REYNOLDS: Yes. So I will preface this by saying it was a fairly -- we were put in a kind of difficult position because before the information became public, we had to tell patients that were enrolled in our trial that they were not allowed to receive more product because an unknown study had been done by an unknown investigator with an unknown product with kind of an unknown result that we really could not explain but that they were -- we could not give them any more drug.

And that was really not a good situation so the FDA has given us some language now which we did pretty much use just as they gave it, some minor modifications, and it goes something like this: "There was a study done in mice in a disease model of a genetic disorder where newborn animals were given very high doses of an AAV vector. It is not the vector that you have gotten. It does not have CF. It is not designed to be used in the clinic and some of these animals when they got to be a year or older developed tumors in their liver.

"Now we do not -- subsequent studies are still ongoing and at this point there is not clear evidence that the vector actually caused the tumors. That being said we need you to be aware of this information. We also need to let you know that first of all that this is not a vector that contains the gene that you have gotten. It is not a vector that was produced to be used in the clinic. It was not given to you by the same route of administration and you are not a mouse."

I should also remind folks that, as Pam stated, our original consent form that went out five years ago when we first started this said, "You know, this is the first time AAV has ever been used in a person. We have very limited data about what this might do to you. One of the things it might do to you is to cause cancer."

And so in my mind we have been up front with our patients and our investigators have been up front since day one. I think we are kind of stuck with presenting them with the current information because it is public domain and they are

going to ask about it and they have already been calling us.

An interesting question is it is really not equal billing. We just saw a bunch of data from many investigators with similar constructs in mice that show no evidence of tumors and we have not included that in our consent form.

I guess one of the things that would be interesting is it is kind of a done deal where it is now. We have a consent form. They have been approved by IRBs and some IBCs. Patient are being -- have been administered the consent form and are undergoing dosing of study agent as we speak.

So I think we are at a place we need to be but I think as more data comes out what would be useful would be to understand what would be the process of incorporating that. For example, if another study is done, let's say replicates this study exactly, and there are no tumors in any animal, can we then take it out of the consent form?

Or if it is proven that it is the disease model and not to the best of our knowledge related to vector, can we strike it then? At what point is it germane and at what point does it remain germane?

DR. MICKELSON: Dr. Samulski?

DR. SAMULSKI: I do not have an answer to the consent form but I have a recommendation. I think the ASGT would serve its community well if they took a summary of this meeting and recommendations and made it available on a website so patients like what Pam has call in can direct them to a layman's version of what was actually determined. Because, as Tom was articulating, by the time you try to describe all this data they are not reading an informed consent, they are reading a documentation of two or three years of experiments.

But the community probably could serve the patients as well as the general public by making some type of analysis of what was done here out to the public and let that be an option that is put on the informed consent that you can get more information about the recent data that was described and what happened at the meeting, and direct them to this. I think this would be a valuable -- and I think that society would also benefit by coming forward regardless of what the vector is or what happens.

DR. MICKELSON: I think there was something on the OBA web page. A background.

DR. PATTERSON: Right. There was some background on the impetus for this meeting and in addition we will be writing up the summary of this meeting as well as the one that was held in December. In fact, I have the draft here of the December meeting. We would confer with FDA that does not preclude ASGT from doing something as well.

DR. SAMULSKI: I just think the informed consent should have access to that and put it on there. Here is where you can get specifically more.

DR. SIEGEL: But I think you -- in addition to any summary that may be planned, you are specifically proposing a summary that is written at a language level appropriate for a consent document, which is variably stated at an 8th or 6th grade level, not presumably the summary we are currently intending. It is an interesting and thoughtful idea.

DR. MICKELSON: You had a comment?

DR. DAWES: I think there is a danger in over interpreting any informed consent agreement because I know how things go once you get down to the patients. You know, well, I have got to tell you this because of the lawyers. We had

this one case of --

DR. MICKELSON: You cannot say that.

(Laughter.)

DR. SIEGEL: I hope you do not say that.

DR. DAWES: No, I mean I have seen things close to that happen. I have been a patient for various things, you know, but there really is no danger. But if I was a patient going in for a procedure I would want to know that there is this data out there, you know. You can say that most -- there is a large body of animal data and clinical trial data which shows no adverse effects, however there is this one unconfirmed report and just sort of leave it at that.

DR. JUENGST: I think there is an interesting use of the informed consent process going on here that I want to flag. We usually think of the informed consent process as disclosing the risks of the research that the subject is being enrolled in. In this case we seem to want to use it to disabuse the subject of misinterpretations of information they have heard in the media or on the internet, i.e. as an educational tool to reassure them that some of the things they have heard may not apply to them.

Otherwise, the kind of consent information we heard from the example seems to be describing the risks of a study which is not the study that the patient is being invited to participate in. That is not required by any moral precepts that you tell the subject the risks of research that is -- is not the research you are inviting them to enroll in.

So if we are going to raise this as a theoretical issue it has to be simply on prudential grounds that, well, they are going to know about it anyway so we better do that for political reasons. Or take the high road, let's do it for educational purposes. They may have heard this in the literature and picked it up and it will be good to just explain to them why the scientific evidence does not suggest that this is a risk of the study. But to leave them with the impression that this is a remote risk of being involved in this kind of study seems inappropriate to me.

DR. GORDON: I just thought -- I feel there is a couple of principles that should guide the way the informed consent should read in this regard. First of all, I do think the information should be given because it exists. I do not think this should be referred to necessarily as a study as much as a finding but I think one of the principles that I feel is being lost sight of in this whole discussion is who is in charge in this arrangement between the research study subject and the physician or the scientist.

I feel that it is the research study subject who should be in charge. If they choose to be hysterical and withdraw from the study that is their choice and I do not think the informed consent information should be presented to them in any way other than the facts as we know it and I think if they choose that that is not enough for them to participate that is their decision to make. I think that we need to be reminded that it is they who are employing us in a manner of speaking just as much as we are relying upon them.

DR. GLADER: I would just like to comment that the hemophilia community at a national level, the National Hemophilia Foundation entirely supports moving ahead with gene therapy studies but their message is go slow, be careful, we trust you.

I think that we have to keep an open -- I view this as a partnership and I think we have to keep -- cannot keep things from them. They have got to be part of everything we know because it has to be an open conscious decision that they want to

partake in this.

DR. MICKELSON: Other comments?

Great. Let's move on to the fourth question then. Should protocols be modified to include targeted screening of research participants for early detection of tumors? And, if so, what would you test for?

And then what tests would be appropriate and the timing and frequency of such tests?

DR. CRAWFORD: And I understand there are no hepatologists here either so a liver pathologist will make a comment. This is a debate that has been going on for my entire professional career and I do not expect it to end and prior to coming here I went to the literature and looked for the latest magic test.

And I was quite intrigued to see a summary from December which looked over the entire world's literature and arrived at the same conclusion that has been there for the last 20 years, which is that simple screening, either by ultrasound or by CT scan or MRI plus alpha protein still remains the method of choice.

So the argument could be made that in a patient population at clear risk, albeit very low, the hepatitis C population, this could be justified regardless of whether they are treated with vector. But it would be a considerable oversight not to include screening in a population that you are enrolling in a clinical trial.

Beyond that it is hard to come up with recommendations beyond what the world's literature currently has, which is interval screening, and I am personally not going to put a time frame down on that but interval screening with tests that are probably best available, albeit it takes a while to detect tumor once it has arisen.

DR. MICKELSON: Dr. Rakowsky?

DR. RAKOWSKY: I guess just a clinical question. What would be the normal course for a grade 2 HCV patient to begin with? Would you be doing yearly alpha proteins from those patients?

DR. CRAWFORD: I am not a hepatologist so I am not the best one to make this recommendation but I would actually counter that by saying what is the clinical course of a nascent hepatocellular carcinoma. And it actually is very slow. On the one hand particularly for a non-cirrhotic patient with hepatitis C, the lead time may be 10 to 20 years or more. And, secondly, even if you have a hepatocellular carcinoma that is not treated, it may grow for five years from a half centimeter up to three, four centimeters before it becomes a lesion that gets into the vasculature and metastasizes.

Now clearly there are always exceptions but, in fact, your lead time to detect a tumor, particularly if you are following it by screening, is considerable. And so, therefore, I would argue that implementation of screening in a hepatitis C positive population that is undergoing a controlled clinical trial or even a noncontrolled clinical trial gives you lead time. It is not going to be perfect but you will have substantial lead time.

DR. MICKELSON: Other comments?

SUMMING UP AND NEXT STEPS

DR. JOHNSON: Anybody else have any burning issues before we sort of wrap this up? Please step up.

I think that as we look at the agenda we have really finished development of panel conclusions because those are, in essence, the answers to the questions. We stayed pretty focused on those and I think the answers to those questions can be transcribed from the proceedings.

I think that the next step for this group is to simply get the working group together to look at the science of this and try to make recommendations relevant for repeating the experiment and moving forward with the science and generating the data.

I think the FDA will consider on a daily basis their role in this and it makes a lot of sense for us to move forward with caution but to also take a look at the science and to support Dr. Sands in his role in these experiments.

So if there are no other questions, do you want to have any closing comments?

DR. MICKELSON: Well, we will be putting the summaries on the web so I think that is something that is part of the conclusions.

DR. JOHNSON: Right. I think that there will definitely be a transcript as Amy said. We will definitely be in touch with a smaller group for the working group.

If there are no other questions, we are adjourned.

DR. MICKELSON: I would like to thank all the speakers and participants. It was a very useful day.

(Whereupon, at 5:19 p.m., the proceedings were adjourned.)

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