On 1/30/07, AJC <ac.aij@xtra.co.nz> wrote:
Timely reminders from 8 years ago from these so-called “services”.
Importance of breast-feeding for conferring lifelong immunity to HPV (among others). Gardisil not needed.
Importance of keeping children out of day “care”
Importance of not allowing govt agencies to separate parents from their children....
etc etc etc
Importance of cronyism in using compulsion or coercion as a marketing tool

http://www.fda.gov/CbER/minutes/0910evolv.txt
10 September 1999

The workshop took place in the Plaza Ballroom, DoubleTree Hotel, 1750 Rockville Pike,

Rockville, MD, 20852, at 8:00 a.m., Regina Rabinovich, M.D. and Martin Myers, M.D., Session Chairs, presiding.

PRESENT:

Regina Rabinovich, M.D.       Session Chair
Martin Myers, M.D.            Session Chair
David Onions, Ph.D.           Panel Chair
John Coffin, Ph.D.            Panel Chair
Philip Minor, Ph.D.           Speaker
James Robertson, Ph.D.        Speaker
Joerg Schuepbach, M.D.        Speaker
Jens Mayer, Ph.D.             Speaker
Thomas Broker, Ph.D.          Speaker
Neil Cashman, M.D.            Speaker
John Sedivy, Ph.D.            Speaker
Frits Fallaux, Ph.D.          Speaker
Michael Fried, Ph.D.          Panelist
Stephen Hughes, Ph.D.         Panelist

Also Present:

Gary Nabel
I N D E X

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1 \hspace{1cm} P-R-O-C-E-E-D-I-N-G-S

2 \hspace{1cm} 8:04 A.M.

3 \hspace{1cm} CHAIRPERSON RABINOVI CH: Good morning. I

4 \hspace{1cm} would like to welcome you back to Session 6,

5 \hspace{1cm} Adventitious Viral Agents in Cell Substrates, and
congratulate all those that were here until 10:00 last night for the latest part of the show, including Mr. Harris, who put in a grueling 18-hour day in yesterday, our visual aides person.

I would like to introduce the first speaker, Dr. Phil Minor, from the National Institute of Biological Standards and Control, who will be giving us an introduction to adventitious agent issues, both reviewing the past and current experience with adventitious agent contamination of biologicals in vaccines.

DR. MINOR: Thanks. Thanks very much. Can I have the slide on, please, or do I just press it here?

What I am going to do is to review firstly all of biologicals, if you like, from an adventitious agent point of view. So it won’t just be vaccines.

In particular, I will be talking about the range of source materials that people have used in preparing biologicals. There will be a clear message that comes
out of that, which is that the more you use well-
characterized cells, the better.

I will also be talking about the SV40 story in some detail, which has been gone through a number of times, but I will be going through it from a particularly regulatory point of view because again, there is a message there which says that if you get it wrong, you will still be working on it 40 years later.

Finally, I will get onto the continuous cell line bit right to the very end.

So there are a variety of source materials that you can use if you are preparing biologicals.

They are sort of listed here, if you like. There are biological materials which are made from whole animals. That would include things like blood and blood products. I will describe that in a moment. So you can just go to a whole animal and take something out and make your biological from that.

You can use your whole animal as a
You can grow material on primary cells. This was the main starting point for things like polio vaccines in the early days, where the SV40 issue arose. Finally, you can grow materials on well-characterized cell preparation. The further down the list you go on this thing, probably the happier you are from the adventitious agent point of view.

This shows some examples of contaminants which have arisen when whole animals have been used as source materials or the origin of the source material.

Most of these will be human rather than anything else, but really an awful lot of the serious adventitious agent problems that have arisen have arisen because of materials sourced from whole animals or using pooled preparation.
The first one on this list here is CJD, Creutzfeldt Jakob Disease, which was transmitted by growth hormone. The growth hormone was produced from human cadaveric material. A very unpleasant disease. It’s almost impossible to detect the agent other than by standing back and waiting for the incubation period to go.

In France, there are still a large number of cases coming through as a result of this. It may well be that around 10 percent are recipients of human-derived growth hormone, will actually wind up going down with CJD in France.

Dura mater is another one. That should be one T, not two Ts. Again, this has been shown to transmit really quite readily when pooled materials are actually used. Almost impossible to detect. Clear, very, very serious kind of consequence of it.

Scrapie was first shown to be a transmissible agent by the use of a TBE vaccine, which
was grown in the brains of sheep. TBE being tick-
borne encephalitis, which then transmitted scrapie to
a large number of the sheep that were actually
inoculated with it. So again, this is a whole animal
source material, if you like, that had quite serious

consequences, especially if you were a sheep.

Over the last 15 or 20 years or so, one of
the best examples of serious or disease-causing

transmissions of infectious agents has been through
human blood and blood-derived materials, clotting
factors in particular. In all of these things, the
entire alphabet soup of hepatitis viruses has been

transmitted by blood product.

In the early days at least, some of these
were really regarded as really a hazard, if you like,
of being a hemophiliac. So, for example, hepatitis C,
in the days when there was a non-A, non-B hepatitis,
it was really regarded as an inevitable consequence of

using factor 8 to treat hemophilia. I am not sure
that that is an acceptable way of actually doing things any more. I am sure that hemophiliacs would agree with that. B-19, paravirus B-19 is still transmitted by clotting factors.

Finally, this one down at the bottom here is a classic example of a transmission by a vaccine, if you like, where hepatitis B was transmitted by yellow fever vaccine back in the 1940s. The hepatitis B actually came from the stabilizers of the albumin that was actually put in there to keep it stable.

There is a story that Fred McCallum, who is head of the Public Health Service in the United Kingdom tells to the effect that he basically won the war because he prevented Winston Churchill having a yellow fever vaccine when he was going off to talk with Stalin around 1944.

So most of the serious consequences really come from whole animal source materials, if you like.

You can use whole animals as substrates. I’m using the term “whole animals” in a fairly broad sense.
Eggs in the definition of the Animal Regulated Use Act in the United Kingdom count as an animal because they are embryonated.

For many years, rabies vaccines were produced in mouse brain or sheep brain. They have quite serious consequences, but not necessarily associated with adventitial agents. You can get encephalitis as a result of immune responses to the non-invasive protein.

The Japanese encephalitis vaccine, which is used for travelers in the United Kingdom, is still made in mouse brain. So it’s not an unusual source of material, if you like. Smallpox for a long time was made on the scarified flanks of calves. Like I said, isn’t any more. However, while these things seem really quite primitive, in terms of how you make vaccines nowadays, you still have a number of vaccines that are made in eggs. Yellow fever is the classic example, and influenza.
Yellow fever is not required to be grown in avian leukosis-free eggs. The reason for that is that there are a number of sites at which it was manufactured throughout the world, where yellow fever is a really very serious problem, such as Nigeria, for example, South America, whatever, where SPF eggs, avian leukosis-free eggs even, were really not freely available. So yellow fever can in principle at least be made in avian leukosis containing eggs, and in fact is. I think there’s no evidence that this has an adverse consequence. But on the other hand, you wouldn’t necessarily want to have a virus in there that you didn’t know about.

Influenza is an actuated vaccine. Again, it’s not made on SPF eggs, that is, specified pathogen-free eggs. They are avian leukosis free, but they are not free of all the other variety of pathogens that you would choose to screen for measles.
vaccine production system, for example.

So even today then you have to bear in mind that a large amount of vaccine that’s made is made on really quite crude materials, from an adventitious agent point of view. It’s not a trivial usage. In fact, when you go through and consider what vaccines are actually made on these days, they are quite primitive, if you like, in some respects.

Primary cultures as been described previously around here, are really cultures that are made directly from the animal. So they are not one pass. They are directly from the animal, if you like.

Here are a number of examples where agents are actually being found or at least located in these kind of cultures.

SV40 is one that I’m going to talk about in some detail in a minute. This was in polio vaccines in the 1950s and very early 1960s, probably, a source from rhesus monkey kidney. Polio vaccines
are still made on monkey kidney, though they are not
usually on rhesus monkey kidney. It would be
cynomologous or something like that, for reasons which

I'll describe in a moment.

Nonetheless, a great deal of vaccine is
still made in primary monkey kidney cells. There are
reasons for that. There's a deep conservatism I think
about changing the vaccine production process if you
have a vaccine that works, largely because you are
dealing with a prophylactic material rather than a
therapeutic material. So you don't want to mess about
with anything if it's reasonably safe and effective.
I'll mention very briefly the defective
retrovirus story in chick embryos. I think Jim
Robertson will probably mention this in more detail,
but I will mention that just as I go by. Finally
recently, the FDA released a talk paper on a
preparation of urokinase, which is used in treating
the heart. This material was grown from primary
cultures made from aborted fetuses. I think it was
aborted fetuses or miscarriages, or whatever. There were quite a variety of infectious agents were actually found in this. I believe this one has now been suspended.

The point is that there are still a large number of materials which are made on really quite basic culture systems, if you like, where adventitious agents are a serious consideration, if you like. So it’s not all continuous cell lines versus the rest.

I mean there are—most of the vaccines that are made in the world probably come from other primary cultures or eggs or things of that nature. I will now talk about SV40. I’m sure in this audience there are people who know far more about SV40 than I do. But nonetheless, I’ll talk about this from what you might call the regulatory adventitious agent point of view, if you like.

So it’s a very common polyoma virus of old world monkeys, and particularly rhesus macaques. The
difficulty with this was that when the rhesus macaque
monkeys are sacrificed and a primary monkey kidney
culture is made from him or her, as the case may be,
a silent infection is set up. So there is on evidence
of infection just by looking at the cultures. In
fact, these cultures can throw out as much SV40 as
they do polio, when you start infecting it with polio.
So you wind up with a culture that’s just stiff with
adventitious agent which you really don’t want.

It’s able to transform non simian cells in
vitro, and it can be tumorigenic if you have the right
kind of animal that you put it into. Between 55 and
62, probably at least a third of all the vaccines that
were made on these kind of cultures, because they were
pooled and the like, were almost certainly
contaminated with SV40. It wasn’t a trivial
contamination. It was really quite a serious
contamination.

Because it was mainly an activated polio
vaccine, there wouldn’t have been that much live SV40 in it perhaps. But SV40 is more resistant to formalin than polio is. So almost everybody who received the shot of inactivated polio in the 1950s, which would include me, would have received live SV40 in some form or another.  

So the concern is really summarized here, which is basically that everybody, I mean this is my own take on it, that everybody—I mean you can argue that it might not have been sort of everybody, but I think it probably was. But almost everybody who received the full course of polio vaccine between 1955 and 1965, also got live SV40 stuck into them. That’s millions of people basically. There were epidemiological studies that were done at the time which really didn’t cause much concern, but they can all be criticized. Some of the studies were really quite short-term, about two or three years or so, looking to see if there were cancer
effects basically, as a result of SV40. It may be
due to the fact that two or three years is not enough to actually find
such an effect, if it actually exists.
The longest which was assumed was over a
period of about 19 years. Most of the individuals
involved in that study would have been oral polio
vaccine recipients rather than inactivated polio
vaccine recipients. So they have had it by mouth
rather than by injection. Again, you could argue that
that might not be the right cohort to actually be
looking at.

So while the studies were reassuring, the
most reassuring thing was that there was no sudden
surge of cancers that you can actually trace back to
polio vaccine usage in the United States or in Europe
where these things were used in a big way. So it
really did seem that in the long term, over about 19
to 20 years, there was no real cause for alarm.

However, in 1992, Michaili Carboni and
colleagues and others, a number of others, including
Janet Butelle down in Texas and the like, identified SV40 sequences which were present in a variety of relatively rare tumors. So myasthenia, which is the asbestos tumor, osteosarcomas, pendymonas, actually the young choroid plexus tumors of children, these sequences do appear to be genuine SV40 sequences.

Where they come from is really not quite clear. Part of the argument was that you could get similar types of tumors in experimental animals, like hamsters. I think that is probably the only example where a hamster is cited as a good model for a human being perhaps. But who knows? In fact, this might actually be an argument that this has got nothing to do with it.

So the question then arises as to where did the SV40 sequences come from. Of course the classic response really would have been it must have come from the polio vaccine because why not?

Now SV40 was discovered around 1961 or
1962 or thereabouts, 1960 perhaps. Directly it was discouraged. There were precautions put in place to exclude it from polio vaccines, because it was known to be a tumor kind of virus, if you like. These were the kind of things that were put in place. They are listed in WHO requirements from about 1962 onwards. They reached their final fully flowered form, if you like, by about 1965. A number of countries certainly had put this in place before that.

The first thing you can do is to use seronegative animals as the source of cells. So you can use animals that have no evidence of SV40 infection as your source. That really is something which is now very firmly in place, which manufacturers now do.

The second thing is, you remember I said that it was the rhesus macaques with the problems. The problem was that the cell cultures didn’t show any sign of having defect, when they were actually
infected with SV40. What you can do then is you can
use species, such as cynomolgus or pattus monkeys,
where the primary monkey kidney culture cell, when
infected with SV40, will actually wrinkle up and die
on you. So at least you know you have got something
nasty and you can throw it away.

Finally, you can test your control of
production cultures for SV40 by the same kind of
procedure. That is why using sesetal cells to see if
anything comes through.

Around the period that this was taking
place, wild caught monkeys were being used extensively
in vaccine production. Up to a half of the cultures
would have been thrown away because of adventitious
agent contamination, mainly foamy virus, but certainly
other things as well. I think that just illustrates
the kind of lack of control, if you like, over the
source materials that was going on, and the extent to
which adventitious agents are really a serious problem
in finding monkey kidney cultures or primary cultures in general.

An alternative way of doing this is to actually use a validated cell bank. Certainly many manufacturers use MRC5, and Mary of course used vira cells, as we heard last night.

Nonetheless, a significant, if indeed not a large proportion of the world's supply of polio vaccine is still made on primary monkey kidney cells, which should really fit this kind of criteria for excluding SV40. One of the questions that then arises is were these precautions good enough? What we did at NIBSC, because we happened to have about 150-odd batches of vaccine archived from the years, was to go back and look at them by PCR. PCR of course is the cat's pajamas. It's really the best technique that anybody ever invented in terms of sensitivity. It's probably about as good as infectivity, at least in our
hands anyway.

But nonetheless, we went back and we looked by PCR at 133 preparations of polio vaccine which had been used in the United Kingdom between 1966 and about 1997. What we had done was looked at all batches of vaccine which had been used since 1980, and all of those were free of SV40 sequences. So that gives you some reassurance that these precautions were actually appropriate.

In fact, the only preparation which had any SV40 sequence in it at all was a seed virus which was used by a manufacturer for making vaccine from. The amount that was in there was around two logs worth of genome as opposed to seven logs of genomes in a really full-fledged infected preparation. So there wasn’t that much in there. The manufacturer had also treated this stuff with toluidine blue, which is supposed to kill of SV40. This was done on the advice of Albert Sabin back in 1960-something or other.

But nonetheless, it does seem to me that
it's rather a foolish thing to have a seed that’s got
SV40 sequences in it at all. I think the WHO
requirements have now been changed so the seed has to
be checked to see if it does have SV40 sequences in
it or not.

This particular seed was not infectious

SV40. We did some quite serious studies on it, like
transfecting the DNA into cells to see if it would
work, infecting monkeys with it to see if we could
actually get seroconversion. There was no

seroconversion. So there was no infectious virus
there that we could actually detect. But nonetheless,
the seed did have material in it.

If on the other hand you look at materials
from around the 1960s or from other parts of the world
a little bit later than that, you can pick up SV40
sequences quite easily. So the method would have
picked it up had it been there.

So our conclusion from that was then that
really as soon as these kind of precautions were put in place, no SV40 would have been present in all polio vaccines used, at least in the United Kingdom and I would guess in the United States as well, because it’s after the same kind of precautions were put in place. So the precautions were adequate. Which means that SV40 exposure of the population through polio vaccines would have stopped around 1962.

So what you then have is the problem of the chorioid plexus and appendinoma tumors, which occur in children who are around two years of age or maybe less. You have to say well how did they get a hold of the SV40 sequences? One possibility, which is mooted with some enthusiasm is that maybe you are getting passage of SV40 from parents who did receive the SV40 contaminated polio vaccine to their children. So how this stuff gets around is quite important.

One of the things that we have been involved in is doing serological surveys of
populations to see who has got SV40 antibodies and who hasn’t. It is about a five percent seropositivity by the assay that we’re using at least. It seems to peak at around age 10 or thereabouts, and doesn’t arise after that.

So what you could argue then is that you are seeing vertical transmission from parents down to their children. What you could also argue is that you are not picking up SV40 specific antibodies at all, and they could be other human polyomas like the BK or the JC, and it’s cross-reacting antibodies that we’re picking up. I think that is still a thing that needs to be resolved. This is how we were trying to resolve it.

We have access to a number of sinomorgous breeding colonies. One of them at least is absolutely riddled with SV40. It’s chronically infected. They are all infected basically.
So this is just four examples of this particular colony. There’s about another 50 or so. This happens all the time. The mothers here are highly sera positive to SV40, all of them. What happens is that the mother and the baby stay together for about six months until the baby is weaned. Then the babies are taken off, no longer being babies of course. They are all banged up together in one gigantic sort of teenage squabbling colony. At the time of weaning, the babies are uniformly negative. So despite the fact they have been on the mother for six months, they have not sera converted to SV40. Almost immediately you bang them up together, or at least within about a month or so, they sera convert. So we actually have a sera conversion panel here, if you like, with about 50 or 100 or so sera, where the babies actually were seronegative and then become seropositive. My view on this is probably that the babies don’t get infected until you bang them together. But it may be that they are infected, but
they are just not seropositive. So what we have to do here is to fish out the virus from these animals here, and see if it looks like the mother’s virus or if it looks like the other babies’ virus.

The point about this long story which I have just been telling you about SV40 is that SV40 was a problem between 1955 and 1962, and it’s now 1999, and we still don’t really know what was going on. So if you actually make a mistake, it’s really quite serious. It may keep you occupied for the rest of your working life.

One last quick thing or two last slides here. One is about reverse transcriptase of vaccines. Dr. Schuepbach will be talking later and Jim Robertson will be talking in a moment about detection of reverse transcriptase in chicken cell grown vaccines, such as flu or yellow fever or measles, mumps, rubella. This appears to be due to the presence of
defective non-infectious particles. There are sequences from EAV and ALV both in these things, a ratio of about nine to one as I understand it. It does seem to me that you are not really quite sure what the AV sequence is in there and what ALV sequence is in there. It’s probably going to vary from chicken to chicken in so far as these chickens have not been bred. In other words, every egg is a new experiment. You are really not quite sure what you are dealing with in that. I think that is quite an unfortunate position to be in. I’m not sure how you control it.

Finally, this is my last slide, and this has to do with characterized cells. The issues that I have been dealing with really have been to do with primary cells and primary cell problems where the virus comes in direct from the animal origin. I think there is no doubt in my mind that that’s the main source of concern in terms of human health. Nonetheless, there are clearly problems
which also arise with characterized cells and the continuous cell lines, in particular. We have some down here.

Now the regulatory authorities in the room will be well aware of a large number of other examples of this type which don’t actually get published. I think that’s not so good. I think this stuff really should be out there in the public literature. But nonetheless, these are the ones which are well known, I think.

CHO viruses, CHO cells have defective retroviruses. Manufacturers take a great deal of care to actually get rid of them in the final product. So they are endogenous.

There are examples of things like BVDV contaminating cells which are growing in culture, and also other bovine viruses contaminating cells in culture, particularly when they are grown on a very large scale. Whether or not that poses a hazard is
another matter, but clearly there must be methods in
place to actually detect them.

The classic example here was the minute
virus in mice, where the tpa had been grown in CHO
cells on a 10,000 litre stove essentially, and then
tiled up for an effect with minute virus of mice. Now
this was on the order of eight logs, as I understand
it, of virus per mil, and yet a 10,000 litre fermenter
culture. This is probably more minute virus of mice
in one place on the planet than has ever been the case
before. You might want to think how you actually get
rid of it actually.

This is a question of actually getting the
cells infected while they are actually burning in
culture. So while family cells are clearly a major
problem, and while whole animal sources, if you like,
are probably the biggest hazard which is likely to be
raised in terms of human health, biological aspects to
do with well characterized cell banks, where viruses
may be introduced from biological materials or they
may be introduced by mice walking across the top of the fermenter or whatever, are nonetheless a significant matter. It really is not totally clear whether these things have an implication for human health. But I think you would be wise to make sure that they are not actually present. That’s where I stop. Thank you.

(Applause.)

CHAIRPERSON RABINOVICH: Please identify yourself.

DR. COFFIN: John Coffin of Tufts. That was a really nice summary actually, Phil. But some caution might be called for in translating the results of vertical transmission experiments from monkeys to humans. As far as we know, simian immuno deficiency virus in monkey populations are not transmitted vertically. Yet HIV-1 is transmitted with reasonable efficiency vertically in human populations.
So there may be some underlying biological difference that perhaps a very subtle one, that promotes this kind of transmission in people, where you wouldn’t see it necessarily in monkey models.

DR. MINOR: Yes. I take you point. We are doing the studies for two reasons. Firstly, to look at natural transmission to monkey on the grounds that it might be a model, although I take your point entirely. But also to supply serum conversion panels so that we can try and sort out specificity of immunological reactions as well. I take your point entirely.

DR. ONIONS: David Onions, Glasgow.

Phil, when people switched to cynomologous monkeys, and I can see the reason because you can pick up SV40. That’s very clear. But how do you know that at the same time, you have not invented a new problem, that you have got another polyoma virus in that species that you are not detecting. I mean has anyone
done redundant PCR to look?

DR. MINOR: I think I would choose to look at a polyoma man to answer that question. Anybody?

DR. MAJOR: Gene Major, Bethesda. In the monkeys that we have used for the human polyoma virus studies, we have screened all those animals for the conventional SV40, but not have addressed the question of whether or not there’s other additional polyoma type viruses that are present there.

Clearly by this time, PCR technology has advanced to the point that perhaps if something were there whose sequences were somewhat similar to the ones that are currently expressed in these animals, we may have picked it up, but we certainly haven’t found anything yet.

DR. LEWIS: Phil, I take it that seroconversion is by neutralizing antibody?

DR. MINOR: Sorry? Say that again.

DR. LEWIS: I take that seroconversion is
by neutralization?

DR. MINOR: That’s right. Yes.

DR. LEWIS: Have you had any chance to look at monkey breast milk to see how long they may be treating antibodian, so that the newborns can be passive immune?

DR. MINOR: Right. The answer is no. We have discussed, for example, getting the urine out of these monkeys after they are banged up together. I am told that actually chasing them around the floor is insuperable. I’m not sure you can actually pry a baby monkey from her mother long enough to actually milk her. It’s a worthwhile question. I think we’ll have another go and see if we can do something about it.

There may be some resistance, however.

DR. BROKER: Tom Broker, UAB. I wanted to follow up on that exact question. We are facing the same problem with potential vertical transmission of human papilloma viruses. I’ll mention it later in my own presentation, but briefly, it does appear there is
some protective immunity during nursing. On the other hand, removal of an infant, say through adoption to another family, is the highest risk factor for a child acquiring laryngeal papillomatosis later in life. So a two to three year delay.

DR. MINOR: So is the assumption then that the infant is infected, but it’s not infected properly then?

DR. BROKER: It’s infected vertically, presumably perinatally, perhaps just before or during delivery, but receive sufficient passive immunity by nursing that gives it life long protection.

So what I would propose as a potential experiment is to literally take the, if possible, take the baby monkey immediately away from the mother, and don’t allow it to nurse, and then just have different lengths of time of nursing to see if this onset of seroconversion is affected by a timing mechanism. Alternatively, don’t ever let that baby monkey be
housed with other baby monkeys in the daycare center,
and keep it with the mother even if it’s not nursing,
and see if it fails to seroconvert.

So the question is, is it getting infected
from its playmates.

DR. MINOR: That’s right.

DR. BROKER: Or is it receiving a period
of important passive protection from the mom.

DR. MINOR: I figured we could do that by
looking at the actual strain of viruses the monkeys
get infected with. We have a number of different gang
rooms, if you like. If you get a different strain in
each gang room, but it’s the same strain within a gang
room, then I think that will answer the same question.

You can also go back to the mother and see what kind
of strain she’s got too. But it’s a valid point.

AUDIENCE MEMBER: I would like to
reemphasize one of the important points that you made.

I know you didn’t have time to expand on it, but I
think it is extremely important. That is the need for those organizations who discover a new virus or some contaminant, cell population used for vaccine production or in a production run, to make that publicly known.

I think that the declaration by Genentech, who has published this information under their name, that an NBM contamination occurred in a 10,000 fermenter is an act of great courage. I think that that kind of courage, this declaration by other companies in this field, is very necessary for the health of this industry.

I understand from some of the remarks that have been made that there are others that are known to a small coterie of people here that have not been publicly declared. I urge all of you to think about this seriously because it can and will have a great impact on this industry. Thank you.

DR. MINOR: I agree totally with that. It
does seem to me that sooner or later the information
will leak out. I think the industry looks very bad.

DR. VAN DER EB: Van der Eb, Leiden. Did I understand it correctly that ferrisfaruses were
found in human embryo material that was used for
urokinase production?

DR. MINOR: I think the FDA can answer this one better than me, yes. But I mean that was my
understanding of it. It’s out on the net.

DR. VAN DER EB: But where does it come from?

AUDIENCE MEMBER: I think it’s a rea

virus.

DR. MINOR: It’s various rea viruses, plus others.

DR. VAN DER EB: I see. Okay.

DR. FRIED: Mike Fried. Was any of the old vaccines from the 1960s that were contaminated,

were they PCR’d up to show that the virus was the same
as being found today? Because it’s also possible that
we all have a latent SV40 type virus which likes to
grow in tumor cells, and that’s why you find it. It’s
a passenger. But I mean since there’s polymorphisms
in the sequence, if you can go back to the 1960s and
then find out if it’s the same thing that we find
today, it would be helpful.

DR. MINOR: We looked at, when you say the
1960s, I have to emphasize this is very early 60s.
Certainly the things that we have got which came out
positive weren’t used in the UK, or they might have
been used somewhere else.

We had a Russian SV40 and we had an
American SV40, and we had an SV40 of unknown origin
all from the 1960s, and they were all different
basically. They were different from the 7-7-7, you
know, the cos kind of sequence as well. So they were
all unique basically, in terms of the region we were
looking at, which was C terminus of t antigen.

CHAIRPERSON RABINOVICH: Last question
MS. MARCUS: Carole Marcus Sequora from Bassey Consulting.
I just wanted to clarify that urokinase is produced from cells. It’s not aborted fetuses. It’s newborns who did not survive. Just for the record.

DR. MINOR: Thank you.

MS. MARCUS: It was rea virus.

DR. MINOR: I’m sorry about that.

CHAIRPERSON RABINOVICH: Thank you. Our next speaker is Dr. Jim Robertson, speaking on experiences with retroviruses in avian and mammalian cell substrates.

DR. ROBERTSON: Good morning. For those of you who don’t know, NIBSC is CBER’s cousin from across the pond in the U.K. What I am going to do is pick up where Phil left off and concentrate on the retrovirus aspect of viral contamination. Initially I will look at say biologicals in general, but
ultimately focusing down on the vaccine issues.

So I will begin with some direct information regarding retrovirus situations with biologicals. I will go onto look at how some of the regulatory guidelines deal with the issue of retroviruses. I will go into look at RTase testing, which is a reasonably current them just now, and finish up looking at the recent situation of the finding of retroviral-like particles in avian cells.

So to begin with, here is a short list of the incidence of retrovirus contamination found in biologicals in general, not just vaccines. I have sub-divided these into two groups here. You see this upper half here, this is where we have in the past had overt adventitious contamination by a retrovirus of a biological. For instance, being mentioned earlier, ALV, that causes virus in yellow fever vaccine by virtue of producing the vaccine in embryonated eggs.
infected with the virus. The other one that was
mentioned earlier by Phil, HIV and blood products.
The bottom half here is a quite, somewhat
separate type of contamination. In fact, you might
find it equaler to call it contamination or not.
Certainly these are not adventitious situations.
These are situations in which an endogenous
retroviral-like particle is present in the
manufacturing process.
In the first instance here, it’s
established that murine hybridomas used in the
manufacture of monoclonal antibodies produced,
secretes C type particles. These have been tested in
a variety of other mammalian, including human cell
lines, and generally are not infectious.
The titre can be very high for these types
of particles. You can get 10 to the sixth particles
per mil. I’ve even seen 11 particles per mil in one
instance. So you can have a very high burden of
3 direct viral particles.
4 It is also well established that CHO
5 cells, which are used for producing
6 biopharmaceuticals, secretes C type particles. You
7 also get intertestinal type particles from these
8 cells. These are probably much more characterized, a
9 bit more work has gone into describing the particles
10 from CHO cells, sequence information from the
11 endogenous elements within the CHO genome, which is
12 producing these particles, give some ideas as to why
13 they are defective. The reading frames are
14 incomplete. There are stop signals. So you don’t get
15 a proper infectious virus from these endogenous
16 elements.
17 The latter type is the only type here
18 that’s dealing with vaccines, even dry vaccines
19 produce either an ovo or cef cells. I’ll come back to
20 that in a few minutes.
21 From a regulation point of view, how do we
22 deal with virus contamination and retrovirus
contamination? There are a couple of guidelines I
would like to bring to your attention. The first one
here is an ICH guideline, which looks at viral safety
evaluation. Admittedly it is only for biotech
products. The scope of the guideline does comment
that this is not, this guideline is not applicable to
vaccines. But I think it is worth looking at what it
says about virus contamination.

Within the document, it describes five
different cases of potential contamination, starting
from the most desirable case, where you don’t have a
virus present in the process in any way, down to the
worst scenario where you know you’ve got a virus, but
you haven’t a clue what it is.

The guideline goes on to state what is
acceptable and what is not acceptable in the
manufacturing process. The only two cases which are
generally acceptable of the first two cases, Case A,
where you have got no virus, and Case B, where you
have got a non-pathogenic retrovirus. The other cases
are only exceptional. Generally you don’t want one at
all. The manufacturing is not allowed when you have
got a virus contamination.

So for Case B, really what you have here
is a murine retrovirus is probably the only
contaminant acceptable in the bulk harvest. If you
remember this guideline is applicable only to
recombinant products and not to vaccines in general,

and these recombinant products are highly purified.

The other guideline is the WHO
requirements which came out recently for use of animal
cells as in vitro substrates. That does include
vaccine production.

When it comes to testing for retroviruses,
this guideline has several—many other guidelines in
the past have indicated, the types of assays being
used for retroviruses, specific infectivity assays,
electron microscopy and transcriptase assays, are the
three general approaches for checking for retrovirus contamination.

There may be the use of specific antigen detection as is in some particular cases, but these are the generally recognized methods of going about picking up retros.

If I can concentrate now on the RTase assays. The traditional type of assay involves incorporating a nucleotide precursor, a labeled precursor of some kind into an assay using a rather synthetic type of template. Then more recently of course we have the PERTs, PB RT, AMP RT type of assays, which includes a PCR amplification step, with the result that these type of assays are incredibly more sensitive than the more, as I can call it, traditional type of assay, and what is often quoted as up to a million fold times more sensitive by virtue of incorporating a polymerase chain reaction.

Now using this type of assay, the cat was
set among the pigeons. When this paper came out, I might even say that the fox was set amongst the chicken coop. Detection of reverse transcripted activity in live attenuated virus vaccines. This quite naturally caused a bit of concern as to what was going on here. The vaccines indicated, the one common feature was that ovine produced in eggs of some kind, measles vaccine out of CEF primed cultures, similarly mumps. Yellow fever and influenza in ovo. But not measles vaccine out of human diploid cells or rubella vaccine out of human diploid cells. So the common link here seemed to be the CEF, the chicken source used in the production of the vaccine. We joined in the boat here and started looking at this issue. Every type of hen fluid that we have looked at, CF fluids or an type fluid from a variety of different strains of hen have all been positive in the assay for reverse transcriptase. Summarily, quail, jungle fowl, and pheasants are positive.
The types of sources of fluids which have been negative for reverse transcriptase are listed here. Some species are not positive, turkey and duck cultures, quite a range of human cell lines. Simian rabbits have been tested and found to be negative. So the clear source of this RTase that was being picked up in the vaccines is quite clearly of chicken avian origin.

We would want to look at—I should add that this RTase was known at the time to be particles associated and appears in the supernate of the cells. We are going to look at this particle to see if it would pick up any infectivity. In all, we looked at 10 different cell lines, mainly human, but including rabbits and turkey. Over 21 tests and 116 passages. In each case, in every test and at every passage level, the cultures were negative for reverse transcriptase activity. There’s absolutely no indication that this particle is infectious. Since
then, CBER and CDC have also come up with similar

data, including use of PBMCs. No infectivity
associated with these RTase containing particles.
Where might these be coming from?

Presumably they are derived from endogenous
retroviral-like elements in the chicken. The
information to date regarding such elements in the
chicken genome are quite well characterized EV loci,
which are related to the avian chosis virus family,
and more recently discovered about 10 years ago, EAV-0
family, which is an older element than EV, and then
older still, ART-CH and CH-1 elements.

The information at the time and pretty
much where it still exists is that we knew that
there’s a line of chickens which was negative for EV.
It had been eliminated from the genome. This line of
chickens, the culture fluids were positive for RTase.
So we knew that it had to be at least one of these
elements producing RT activity. At the same time, you
couldn’t eliminate the fact that EV might also be
producing RT activity. The best bet was EAV-0, given
the sequence information that was present at the time.

More recently, in the last year or two,
Joerg Schuepbach’s laboratory has produced a good
evidence for the presence of EAV-0 derived RNA
associated with the RT particles secreted from CEF
cells, and then this year, Walid Heneine, CDC, also
produced the presence of EAV and ALV RNA. When I say
ALV, I mean derived from the EV loci and not exogenous
ALV contaminating RNA.

So what can happen here retrovirus-like
particles, defective particles being produced from
endogenous elements both from EV and EAV-0 family of
endogenous elements. The presence of the RNA and
reseqnstrictase in a particulate fraction leads one
to come to the conclusion that we have retroviral like
particles in the CF fluids of the chick cells, which
is present in the vaccines measles and mumps.
The absence of infectivity in the current genetic information, sequence information that we have on EV loci and the EAV-0 family of endogenous elements strongly indicates that these particles are defective viral particles. The only question mark that remains from the regulation point of view, but also scientific point of view, the possibility of pseudotype formation during vaccine manufacture. The current evidence suggests the particles that are defective in the envelope-like protein and so there’s a particulate of pseudotype formation with the glycoprotein of vaccine viruses being grown in the CF cells.

So to summarize a couple of these issues then, from the practical point of view, testing for reverse transcriptase as an indicator of retroviral contamination, these assays are evolving, changing all the time. One has to take into consideration the strength of the assay and the validity of the assay. There may be different requirements within an assay.
for different sources of RT. It may be necessary to
use some other sort of method to assess the
significance of any RT detected because we know that
RT activity can derive from other enzymes. Telomerase
is or DNA polymerase, cellular DNA polymerase is.

These features are not specific to the more recent
sensitive type of assays involving PCR, the parents,
and the PBRT. These features were also factors that
had to be considered in the more traditional types of
assays.

It is often quoted that the RT levels in
chick cells is very low, given that it was detected by
a very, very sensitive assay, and has not been
detected by the more traditional type of assays.
Certainly some preliminary data that I have got
suggests that it is not quite as low as we first
thought. Really this RT activity in chick cells and
ultimately in vaccines is only just below the level of
detection of the more traditional type of assay. In
fact, this was a relatively novel phenomenon
discovered just a few years ago. It was in fact first reported 20 years ago in the late 1970s by Berne and Hofschneider at the Max Planck Institute in Munich. They reported the presence of a novel type of RT enzyme in chick embryos and in chick cells. That was in the days before PCR.

So the level—I certainly believe the level of RT and the level of these particles is actually quite high in chick cell fluid. Ultimately I think what we have to do is look into the need for standards, standard materials in some way to assess on a quantitative basis the level of RT activity in chick cells, in measles vaccine, in mumps vaccine, in order to come up with some meaningful conclusions regarding it.

So to look at the RT issues from a regulatory point of view, a couple of comments I would make. First, that these are state of the art technologies. When these highly sensitive assays
first came about, it posed very useful from a research point of view to what use are they in a routine manufacturing validated type of assay. I think the time has come where yes, you would say that these are state-of-the-art techniques and can be and should be used for detecting the presence of RT in your manufacturing process. However, when it comes to, for example, chick cells, and until we have a greater understanding of what the levels might mean, and until standards are available, there is really—it is difficult to justify any requirements to perform RT or PBRT assays on systems, and basically here I am talking about chicks, which inevitably will be positive. We know they are going to be positive, that there’s no great need to actually require any manufacturer to do these assays. But certainly there is a still a strong requirement to provide evidence for freedom from retrovirus contamination. This will have to derive from other
data. Thank you.

(Applause.)

CHAIRPERSON RABINOVICH: Thank you, Dr. Robertson.

Any questions?

AUDIENCE MEMBER: Just a comment. For known endogenous avian retroviruses or exogenous avian retroviruses, of the cell lines, of the cells that you tested for infectivity, only the turkey cells would have given a positive result. I would urge for avian-derived—urge the use of those cells, and a PERT assay is a sensitive readout, for detection of perhaps unknown agents in these vaccines, end products, as being the most sensitive, at least for avian-derived.

DR. ROBERTSON: Yes, yes. The turkey cells are sensitive for rav, because it's virus, but apparently not for the RTase. I have no idea, duck cells are also negative, but I have no idea if duck
cells are susceptible to the --

AUDIENCE MEMBER: They are not as good as
turkeys. Turkeys themselves actually are not
sensitive to all exogenous ALVs, but to most they are,
and all endogenous ones.

DR. ONIONS: David Onions. I really
enjoyed that, Jim. I just want to make a comment on
your comment about standards. I think as we heard
from Keith last night and what we’re doing, and I’m
sure George is doing too, using the tac man technology
where you can actually quantitate the PCR product.
Then if you actually do EN counts of virus particles,
dilute these out, you can actually quantitate your
assay system and actually determine the number of
particles you can detect.
Now it seems to me that that is a useful
kind of standardization, and that you can then relate
that to if you like, a consistency of your starting
material, in this case the egg.
So I think in that case, applying those
techniques does have value, because it gives you a
kind of lock-to-lock consistency of your materials.

So that if something goes out of spec, then perhaps something odd is going on in those materials.

DR. ROBERTSON: Whatever we want to approach this standardization, one would have to do it on a quantitative basis. I was sensitive earlier to quote any actual figures, but to quote you some figures that I have got so far, in one chick cell preparation, the culture fluid, there was the equivalent of the order, and this the first of investigation, 10 to the 4 focus forming units of rav in uninfected chick cell fluid.

A large current high level is when your typical infection goes up to 10 to the 6th, I believe, focus forming units. You are only talking about 104 drop, lower value. So you are not far away. It is going to be difficult. If you have got an overt contamination going up to 10 to the 6th, I think that
will be quite clear on a quantitative basis. But once
you drop down a bit, it is difficult to say whether
you have got an infection or whether it’s just
background level of endogenous RT-derived activity
that you are picking up.

DR. MYERS: Martin Myers from National Vaccine Program. As I sit and count the number of
immunizations that various populations receive with
these particles in it, repeated immunizations with it,
I wonder if there is any data on sero responsiveness
in longitudinal --

DR. ROBERTSON: Well the reason I am
looking over your shoulder, we have Walid Heneine from
CDC. I’m not sure if you are going to say something
along those lines, but serologically, there is no real
evidence for reaction to ALVs. Epidemiologically,
when it first came out, we also, not ourselves but
epidemiological colleagues, to provide information.
There is no evidence again, for any increase in the
incidence of childhood cancers since the onset of measles, mumps vaccination.

Walid, were you going to say something?

DR. HENEINE: Yes, I have. Regarding transmission risks, so far the data we have where we have looked at the presence of antibodies to avian leukosis virus by western lot, I’d say we developed as well as presence of ALV sequences and EAV sequences in the peripheral blood lymphocyte from vaccinated kids as well as in plasma. So far, the results have been all negative for both viruses.

I have just one comment regarding referring to these viruses in general as defective.

My comment is that the evidence we have so far on those we have studied in a couple of vaccines between in Europe and in the U.S. suggests that those, for example, ALVs we’re dealing with could be defective because they come from loci that have deletions.

However, this may not be true for all the contaminants
from it we might find in other vaccines, because these contaminants reflect the particles expressed from these loci in the different cell substrates that Phil mentioned in his talk, that the nature of these particles and their phenotypes would vary depending on the presence of the particular loci in these substrates.

So just a comment, not to generalize that we always should expect to have defective particles.

We might or we might not in certain cases.

DR. ROBERTSON: Yes.

DR. SCHUEPBACH: Joerg Schuepbach from Zurich. I would also like to make a comment regarding seroconversion. We have done two vaccination studies, one with yellow fever vaccines, where about 120 individuals were tested actually for reactive antibodies against HIV. The reason for this was that in 1991, there have been reports that false positive HIV reactions were found in people that have been vaccinated against influenza. Influenza vaccine is
one of the vaccines which contains the EAV.

So we found that statistically highly significant the vaccinees which have received the vaccine which have the higher content, about 80 times higher than the other vaccine which we used to use too. That these patients actually had highly significantly elevated antibodies to HIV 1 and 2, although none of these actually became sero positive. The serious bonds was highest in those individuals that also had a history of measles vaccine.

In the second study, this was an influenza vaccine where we tested two different brands, a split vaccine and a rather crude vaccine. We also had a response to HIV bond two in the third generation tests in the vaccine which contained more in the crude vaccine, which contained more of the EAV protein, and again those individuals who had a history of yellow fever vaccination had the highest type, the highest increases.
We also tested yellow fever vaccinees by PCR, RNA PCR and DNA PCR for EAV-0 sequences. We found one out of 180 individuals in which both these tests were positive as plasma on PBNC. At the moment, we cannot exclude that this was the result of a contamination, but we are working on that. So I think that the matter is actually not as clear as has been presented by others.

MS. SHEETS: Hello. I’m Becky Sheets from FDA. How would you recommend that avian-derived products be tested for retroviruses? EM is not very sensitive. The conventional test is often inhibited by the allantoic fluid, and therefore, is not necessarily a valid test. How would you recommend, if you don’t use a PCR-based RT des?

DR. ROBERTSON: At the end of the day, it would have to be an infectivity assay. There are also some antigen, ELISAs for the viral antigen. I don’t have experience with those. I don’t know the
sensitivity of them. Ultimately you are looking at an
infectivity assay which can be performed even on chick
cells, which are positive for RTase. One could assess
for after several passages on chick cells, looking at
an increase in RT activity or increase in antigen.

MS. SHEETS: When you said infectivity
test, were you talking about those specific for ALV or
were you talking about general tests to detect any
kind of retrovirus?

DR. ROBERTSON: Well, it would have to be
an avian retrovirus if one is performing the assay on
chick cells. Propagating the material, the test
material in chick cells but using either RTase in
general or an ELISA specific for ALV test for
increased presence of either RT or for the presence of
ALV antigen.

CHAIRPERSON RABINOVICH: Dr. Coffin, the
last question?

DR. COFFIN: Yes. I’d like to actually
address Dr. Schuepbach’s comment. Did I understand
that you were basing your sero assays on the rationale
that there might be cross reactivity between ALV and
retro viruses in HIV? There is no rational basis for
that. There’s virtually no amino acid the same
between those two viruses, except for some extremely
highly—you know, three or four in pol and some
other places. Did you actually assay directly for
seroreactivity against ALV? It would have been a much
more straight-forward experiment.

DR. SCHUEPBACH: We agree that there is no
sequence homology on the nucleic acid and on the
protein label, but these are the results which we
found. We have to find an explanation for them. We
don’t have at the moment.

CHAIRPERSON RABINOVICH: Thank you, Dr.
Robertson.

Our next speaker, Dr. Jorg Schuepbach,
from the Swiss National Center for Retrovirology.
Induction/activation and detection of occult viral
agents that are present in mammalian tissues.

DR. SCHUEPBACH: May I have the first slide, please? Okay, from previous remarks, I heard that I was expected to talk about these avian retroviruses as well, but actually I was asked to talk about the induction activation of occult viral agents.

So I will just have a few remarks on this other stuff.

So occult viral agents are agents you don’t detect or at least do not easily detect. They may include two groups: a group that includes known agents which are present at two low concentrations for easy detection. The reason for these may be latency;

The other group consists of unknown agents. Since we do not have good detection methods for these, they may be present at low or also at higher concentration.

Viruses known for their latency or various types of the herpes virus, true, they are latent in various types of non-permissive cells such as neurons, B cells, monocytes, PBLs, and others. They are
activated from these latent stages by various kinds of 
stimulation of their host cells by differentiation, 
agents by the differentiation of precursor cells, to 
more mature cells. Again, by other activating agents. 
Other viruses could be considered in 
addition to the herpes viruses include the adeno 

viruses, the adeno-associated virus and the pathyloma 
and polyoma viruses of which we heard yesterday, and 
will hear more in a subsequent talk. 
Regarding the RNA viruses, I might discuss 
the measles viruses and of course the retroviruses. 

When we look at the mechanisms by which we 
can activate these various viruses, it is mostly by 
activation of their host cells, by cell stimulation, 
by induction of cell differentiation of these cells, 
and then by co-cultivation with cells which are 
permissive for replication. 
Now since we have different viruses and 
host cells systems, these methods vary greatly among
the different viruses. If you have unknown viruses, you really don’t know what to do.

So the effect of such activation would be that from a lonely latently infected cell, by inducement of replication, a virus would spread throughout the culture, resulting in virus gene amplification in production of viral proteins. So this would make of course the detection easy. You might also have some pathogenicity which is easy to detect.

However, our goal is actually not detecting any possible virus that might be present.

The principal goal is to provide a virus production system which is free of such agents. It is suggested here the easiest way of achieving this is actually cellular cloning. Because if you have an agent that is present in only a minority of the cells, the chances that you derive a clone that is free of these agents is very high.
If by chance you hit an infected cell, the

descendants of that cell will all carry along the

test and of course then we come into a situation

which makes detection of unknown viruses and also

known viruses much easier because either all of the

cells will be infected or none at all.

So cellular cloning, if we hit an infected

cell, has actually a viral gene amplification effect

which is comparable to virus induction activation if

it’s successful. Most importantly, it is a procedure

that works for all the latent viruses except

endogenous retroviruses, but these are present anyway

in all of the cells.

So going on to the detection methods for

test agents, let’s first talk about known viruses.

Since all the cells will be infected, we actually do

not need the most sensitive procedures. We do not

need procedures that detect the single viral copy.

What we need is broadly reactive methods which go
detect all the different members of a certain virus group.

So I think techniques, old-fashioned techniques like hybridization techniques on the low stringency or if we want to use PCR or nucleic acid based methods, we should take care that we take a lot of different probes, use data generated primus, multiplex PCR and so on.

Of course in addition, we should also do the classical methods, doing cell activation and co-cultivation as permissive cells, the routine detection methods of broadly reactive antibodies which detect all the different members.

For those who think that what I have told so far is rubbish, and that we actually do need very sensitive methods, I offer the mega PCR, which has also been named catcher PCR by others. The purpose of this method is to take very rare sequences among a very high background of DNA or RNA. So here we convert the samples of up to 500, maybe even 1
milligram of DNA or respectively RNA.

The principle is very simple. We use biotinylated capture probes which bind to these sequences inquest. We isolate these complexes on coded beads, wash the rest of the DNA away, and then amplify these by PCR with primers which are located outside of this capture probe.

The advantage of this is that we absolutely do have no carry over because the amplicons are selected against when we do the capturing. It is this type of test which I would actually like to have been seen when testing in the question of xenotransplantation where the PERV sequences can be found in humans which have received pork material. I think this will be the test, to test these questions.

Now using this method, it’s actually very sensitive. You can detect a single copy here of HIV DNA. We still have double positive signal, is about one copy. This serial dilution was done in the proper
range here. The fact that in these two, three last
dilutions only one of the two duplicates was positive
clearly demonstrates that we are in a Poisson
distribution. So we can detect the single copy with
this method in 100 microgram.
DNA, we have actually demonstrated that
there’s 95 percent probability we can detect three
double standard HIV copies in 100 micrograms of DNA.
So now going on to the exclusion of
unknown viruses, and I will talk about retroviruses
later, we can actually use the same procedures as I
have already described previously. We just have to
take care that we really have broadly reactive
methods. This is true for molecular based tests as
well as for the more classical procedures.
Now coming to retrovirus detection, of
course also of cell cloning, here we have two
situations, the exogenous retrovirus may not be
present in none of the cells or in all of the cells.

The endogenous retroviruses were always present in all of the cells. The known exogenous retroviruses are detectible by tests for conserved sequences. Of course you might also use universal pool primers for unknown retroviruses—because of the endogenous retroviruses. Not all of which, or very few of which are actually harmful.

So I think it is better at this time to switch from the analysis of cells to the analysis of particles. This is best done by the PERT assay which has been mentioned before by several speakers.

Now when we devised this test in 1992, we devised it as an anti-family of related tests which would have in common that reversed inscriptase present in a sample would be used to create from a template primer combination and nucleic acid that is to be unamplified.

Now in most instances, this will simply be
the cDNA. There are other possibilities as well. You can take any nucleic amplification procedure, not just PCR. You may also use ligase chain reaction or NASPA or you can make use of auto replicated DNAs or RNAs in order to generate amplification product, which can then be assayed by different methods.

So since we have provided for all these different methods already in 1992, we do not think that it is necessary to invent new names for these current assays.

Now this test is actually very sensitive. This experiment in comparison to classical RT assay. It occurred as six to seven orders of magnitude more sensitive, and in a direct comparison with—in the case of HIV, where we compared the method with RT PCR, detecting one copy of cDNA, we had the same dilution endpoints for two different samples.

Actually as others, we can detect only a few particles in the case of HIV. We believe that in some cases we can detect even less than one particle.
Now this is one of the theories taken from the Joerg Koenig paper in 1996, where we demonstrated that the measles vaccines, the mumps vaccines, the yellow fever vaccines, and the MMR vaccines all contain activity which is about three orders of magnitude higher than the background here on other vaccines, and were negative.

Now in order to identify the viruses behind these activities, we along with the PERT assay, developed the method for the identification of unknown retroviruses. It is based on three properties of old retroviruses, namely, that they all are polyadenylated, that R sequences are repeated at both ends, and that cDNA synthesis has started here at the primer, binding site, and that for primers, tRNAase are used and the use of such tRNAase is actually very much restricted among the various retroviruses. For example, is just four PRNA primer equivalence. You can start cDNA synthesis for all exogenous
17 retroviruses known today.
18 So what we do is that we bind the
19 retroviral RNA to poly t coated beads. Then we start
20 here, the synthesis of the cDNA with one of the
21 various t RNA primers, synthesizing the strongest of
22 DNA. Then adding a tail here, and then with anchored
23 TCR, we can amplify this sequence and submit the
24 sequencing directly.
25 Actually this method has also been used by

59
1 the group of Dr. Loewer at the Paul Ehrlich Institute,
2 and even published before us. But we have somehow
3 optimized this procedure, so in general we need less
4 than one-thousand RNA sequences, sometimes as few as
5 20 or 40, 50, in order to generate this sequence here.
6 As soon as you have it, you actually know whether you
7 are dealing with a retrovirus or not.
8 When you deal with a retrovirus, you have
9 to R sequence and then you can check with the other
10 anchored PCR. Where there is R here, it’s repeated at
the three prime end. If it is, you can then amplify
the entire genome with a little bit of luck by long
PCR.
So this is what we use to identify this
EIV-O sequence. We have also done some other work.

For example, we investigated the NIH 323 cell line.
This was negative by convention RT tests, but positive
by PERT assay. We had a nice band in sucrose, and
then radiant. Using this procedure which we call
parar, we identified 23 different products, 15 of
these were actually retroviral sequences from four
different groups. Three of them were unknown
sequences, at least at that time. So far we have not
further characterized these sequences, but this is
still awaiting.

Now staying with retroviruses, as Dr.

Coffin pointed out yesterday, sometimes if you have a
cell line here, you are dealing with melanoma cell
lines which were found to be highly perciipated by PERT
We analyzed what was in there. It turns out to be endogenous murine leukemia virus, and later we were told that these cell lines have actually been passaged in mice.

If you have low titres of activity, then that becomes a little bit more complicated. This is the analysis of primary samples from a patient with MC cor cultures. No actually not cor cultures, just cor cultures which were found lowly positive in the PERT assay with activity in the order of two, maybe three times above background.

Here the patterns is a little bit more complicated. You have here a small peak that might correspond in density to ritualized particles. This one might correspond to cor particles. You have another identified—unidentified peak here. It will certainly be a challenge to find out what this stuff is.

Next, please. This is another example of a primary culture where we have a very short peak at
the higher density. This might be for particles,

could be a different retrovirus, a different virus, or

just a subcellular particles containing some cell or

enzymes.

Now you will say that this test of course
detects only retroviruses that are released. We are

also worried about retroviruses that are inside the
cells, so stimulation may be necessary. Actually I

think one important question is or one possibility is

that actually the vaccine virus we would like to

produce in such a cell might activate latent

proviruses. So I think it is important that we

actually do not just test the virus production systems

while uninfected, but also when this seed virus has

been added, and then we harvest the virus.

Now in some cases, as in the measles virus

or so, this has proven very easy. We had quite a good

specificity. But in other cases, it might be more

difficult as indicated in this example, where we
tested a vaccine, experimental vaccinia, recombinant vaccinia virus vaccine against melanoma. This was found highly positive by PERT. It had actually been produced by just the lysing, the infected cells by ultrasonication.

What we now find is here in black, is the vaccinia virus DNA two peaks. We have here a major peak of RT activity which does not coincide with the vaccinia virus peaks, and also is not characteristic of retroviruses. So I think in this case, we can rule out the presence of a retrovirus.

Now it may also be interesting to find out whether upon induction, viruses might come out. So this would add an increase of safety to the vaccine. As retroviruses are regulated, you have the promoter in anti sequences in the upstream LTR in the U3 region. Depending on the cell type, activation state of the cell and the differentiation, you have various sets of transcription favors interacting with this
enhancer regions.

In addition to this balance of positive and negative transcription factors, you may have positional effects as the chromatin structure or the DNA methylation. You may now try to influence this balance by tipping it by either inducing mitosis cell differentiation by substances that lock inhibitors or by alleviating the negative positional effects, again by inducing mitosis or by inducing DNA de methylation.

The number of induces have been described in the past. The most important ones are listed here at the top, allogenated pyrimidins, the azacytidine, which only both of them working only in infected cells. I will not mention the others because of the lack of time.

Now it depends a lot on the virus whether azacytidine or the deoxy pyrimidine is preferable. For example, in experiment in cell line where two types of different retroviruses are produced, several
type A particles here. The azacytidine is certainly better. But in C-type particles, these cells produced IdUdr. Yes, the IdUdr is better. So you might have to use a combination of these two drugs.

So in conclusion, I think induction activation certainly serves to amplify latent viruses for which improved detection. I think it is more important that we early in the process of selecting virus production systems be cloned B cells, and sub-cloned, because this will amplify, because this really facilitates detection very much.

In consequence of this, we do not—I think this is very important. We do not need the most sensitive procedures. What we need is broadly reactive procedures which will detect all the different agents.

I also think that at the end, the only important thing actually when dealing with adventitious agents, not just with DNA, which might be
infectious, is that the vaccine is free of these contaminant viruses and for retroviruses I believe that this can be verified by the PERV assay. Thank you.

(Applause.)

CHAIRPERSON RABINOVICH: We’ll take just a couple of questions because I would like to leave the rest for the panel discussion.

DR. COFFIN: John Coffin. I would agree that if you get preparations of vaccines that are negative by all these assays, you can have a pretty good level of confidence that they are not contaminated with retro viruses. The problem is, if you do these enough, it may well be that no vaccine will pass these tests.

What I think is very important to add to this would be one more level to your last slide. That is an infectivity step. As in the example we saw before when one perhaps collects a panel of cells or cell lines which are pert negative, and there seems to
be reasonable numbers of those, and then test the
vaccine, the induced stuff and everything else by
infectivity and induction of pert activity on those
cells.
I think that would be a much more useful
and reliable test for the presence of viruses that
might be problematic than simply looking at the pert
activity in preparations with cell soups.

DR. SCHUEPBACH: Yes, I agree with you.
I actually thought that was included in those
conventional methods which I have listed for the known
viruses. Of course you should also do some studies
for retro viruses.

AUDIENCE MEMBER: You mentioned results
associated with particles from supernatants of primary
human materials. Did you try to find retro virus-like
sequences in these particles for para assay?

DR. SCHUEPBACH: Yes. These are very
recent results. We are in the process of doing that.
One of the issues in testing vaccine products is obviously what tests are available and have been validated and that we understand the sensitivity of. So I guess in the context of thinking of highly conserved sequences to which we might develop primers that could detect a broad array of viruses, including some unknown related viruses, what can you say about the current state of the art? How good is that? How well has that been validated? Is that something which if we decided tomorrow we wanted to apply that to new vaccines producing neoplastic cells, we could simply say “let’s do it” or is more work required?

I’m actually not very familiar with other viruses than retro viruses. But I think these things, however they exist, should clearly be developed.

Thank you. We will go onto our next speaker, Dr. Jens Mayer, from
the University of Pennsylvania. The status of HERV in human cells.

DR. MAYER: Okay. My talk will deal with -- can I have the first slide, please? Okay. My talk will tell you something, I hope, about the status of these human endogenous retrovirus regarding the coding capacity and the expressions. Just again, it was mentioned before already what is actually an endogenous retro virus. HERV is created by the germs of infection of an exogenous retro virus. This leads to radical inheritance of this newly created virus following generations. In the course of the evolution, it will be also inherited to newly arising species.

The human genome, like all mammal genomes, and also some invertebrates, invertebrate genomes where it has been shown, contains several families of elements and so on. It has been estimated that about
one percent of the human genome of such retro origin.

These elements antiquated already several million years ago through the genomes of human predecessor species. Some present for at least 30 million years.

Some have been shown to be present for at least 40 million years. We have several indications of different various families. So they were independent of several exogenous retro viruses. Some of these elements that are now present in the human genome existed. Single copy, and some have copies, copy numbers up to 1,000, per haploid genome.

But as I said, most of these sequences were already present for a long time. Therefore were targets for mutations. Most of these families then became coding deficient or they do no longer encode for retro R proteins. However, even if they are coding deficient, many of these families are still transcribed in several human tissues. Some have been discolored just by virtue of their expression.

It also seems that the expression of these sequences is regulated in certain tissues and tumors,
so we heard that there might be an deregulation of families. It seems possible that that deregulation mechanism is not present in certain tumor tissues.

Just a word regarding the nomenclature of these sequences. The tRNA that was originally used in the priming of the transcription process, the life cycle of the exogenous vaporized, and according to the amino acid and tRNA codes for, and this single code for the amino acid stands dependent. This is just one possible nomenclature of perts. It’s still very confusing.

I said that most retro viruses are coding, HERVs are coding deficient. However, there are some good described examples, especially some new examples of coding in tact HERV sequences. At least there are some in tact genes. We have already known for a long time the so-called ERV-3 sequence that belongs to the R-family. This, we agreed, pro-virus, or pro-virus
sequence encodes, and 1.9 KBN open reading frame.

That open reading frame is highly regulated to the
transformation of trophoplasts into sensitio-
trophoplasts in the placenta. So we have here clearly
an up-regulation during a developmental stage.

We have for instance, you have H-family
and we have about 1,000 copies of that H-family.

Among them are 100 copies that are still in tact
regarding the pro-virus structures. They have an LTR
gag pol env, LTR structure remaining 900 lack N gene.

There has also been reported that this HERV-H families
are expressed in various cell lines. We see the
highest expression for these elements has been
reported in cell lines that are derived from germ cell
tumors, and germ cell tumors I guess you will hear
some more about germ cell tumors later on.

Just this year, Lindeskog, Mark Lindeskog
reported the isolation of an intact HERV-H env gene.

So it is now clear that there is within the human
genome one intact HERV-H env gene. It’s not know so
far whether there are any among these many sequences,
whether there are any intact gag of pol sequences.

I would like to mention the new discovered
HERV-W family that has originally been reported, has
been isolated from retro virus by particles from
multiple sclerosis patients. It has also been
reported that these HERV-W sequences are up-regulated
in the placenta. Joni Blanc also reported this year
the isolation of an intact HERV-W in the genes. It is
also not known whether there are intact gag pol genes.

I would like to in the second part of my
talk, report about results for our family of clearly
outlines from our other HERV families in the coding
capacity. This is the so-called HERV-K HML-2 family.
This is quite complicated.

The human genome contains several families
that use lycine primer binding site or TRNA for primer
binding. They were named human MMTV-like sequences,
one through six. The family that we are talking about is reported in more detail by Ono and co-workers and the original sequence was the so-called HERV-K 10 sequence, which is by the new nomenclature is the HERV-K HML-2 sequence.

We have reached about 25 to 50 copies of that HERV family is present in old world monkeys, but not in new world monkeys. One concludes that family is present for at least 30 million years in the genomes.

In the past, there have been reports of isolation of the isolations of intact HERV HML-2 sequences. So there were reports about intact gag sequence and intact protease sequence has been reported, that is able to process that HERV-K gag protein, intact pol sequences with RT activity, with endonuclease activity, and have been reported and also intact mRNA has been reported from the group from Johannes Loewer. And also what we heard yesterday evening, there is also an additional splicing product
from the N gene, the so-called C-ORF that still has a rav-like function.

What is known already for a longer time is that these particles or the cell lines are derived from germs of tumors or typically testicular tumors of the young man. These cell lines do produce with rav particles. Boller and coworkers could show that these particles are encoded by the HML-2 gag protein, labeled antibodies, and recognized that gag protein. If we look at patients suffering from germ cell tumors, we also have some surprising results regarding that HML-2 sequences. Namely, if we look at the antibody status of these patients compared to controls or other non-germ cell tumor types, we see that mixed germ cell tumors and here especially, seminomas, these patients have very high antibodies directed against HERV-K gag and HERV-K N proteins.

These tumors or these antibody titres are already very high if the tumor is clinically detected. From other
results, we also know that the precursors of these
tumors, the so-called carcinoma in situ, also
expresses already on the RNA level these HERV-K HML-2
sequences.

We were interested to see or to find out
where in the genome are these impact genes located
that are responsible or that cause finally the
production of these gag and env antibodies. As I

said, it has previously already been reported that
they are intact genes, but it was not possible because
of the high copy number of these sequences to isolate
or to at least chromosomally assign these intact
genes. We, therefore, tried to chromosomally assign
these intact sequences using a combination of the so-
called protein truncation test and using a
monochromosomal hybrid panels, or panel of human
rodent fusions, fusion cells.

We were able to show that there are at
least, still at least eight intact gag genes within
the human genome, and at least three intact env genes.

We did not publish that. There are also several intact pol genes within the human genome.

I just want to show you how we got these numbers. This is the protein truncation test that has been described by Roest and coworkers in 1993. So it was originally developed for the detection of APC gene carriers that carry it, the APC gene. So the APC lesion is characterized by trends or not completely translated APC proteins. It is almost like the 3 prime terminus.

So we have three possibilities. One is that both are intact, both genes are intact. The carrier will carry one defective APC gene. The defective person would carry the two defective genes.

The principle of the test is that the coding sequence is PCI amplified, where the protochomo contains the T-7 promoter and the translation initiation sequence.

So if this PCI product is then in vitro transcribed
translated and impressed and radiolabeled amino acid, electrophoresed, and then auto radiographed, you will see according to the status of these donors that you will have only full-length proteins, the carryover also show an additional shortened protein and defective people will only produce defective proteins.

We in principle used the same test because we in principle have the same situation. We have some defective gag genes within the genome. There must be at least one gag or env gene because we have the antibodies. So we put—in principle used the same test. What we did was we are looking for the presence of full-length gag genes or env genes on the human chromosomes and then tested the PCI product we got from the chromosomes for their coding capacity. This is the result for the gag coding capacity. So gag protein would result in a protein of about 73 kilodaltons. So these are controls that give the respective proteins.
You see that there are several human chromosomes that contain or produce a full-length protein. There are eight human chromosomes that contain at least one gag gene that contains four full-length proteins.

I also would like to mention that we are also able to demonstrate the defective gag genes if we see here, these proteins that are just smaller than expected. These are very likely the gag genes that are defective. Stop codons within the coding sequence.

We did the same for the HERV-K env genes. We see here that three chromosomes produce a protein of about 76 kilodaltons. These are the chromosomes 7, 19 in here on the chromosome.

What we also see in the gag experiments is that there are additional env genes that are only on the almost intact. We have here a protein that is about four kilodaltons more. So this actually could
also be considered as an intact reading frame.

So we have several human chromosomes that still contain gag and env genes. We have three chromosomes that contain both intact gag and env genes, the chromosome 7, 19, and the Y chromosome. We were interested whether these chromosomes or the intact genes on these chromosomes are derived or located within one provirus or within several or different positions within the particular chromosome.

I would like to report or tell you something about what we found out for the chromosome 7. We were using for addressing that question, we were using a chromosome-specific, chromosome-7 specific cosmid library. We were screening for clones that contained both gag and env sequences.

What we finally found out, that we isolated the so far least defective human endonuclease on chromosome 7. We were able to characterize the proviral sequence within one cosmid clone that still
has intact LTRs. So they regulate to the elements.

They are able to transcribe, as you will see. We have an intact gag gene. We have an intact protease gene that protease is able to cut itself from a gag protease, polymer precursor protein, and is furthermore able to process encoded gag proteins. So it’s typical retro-ized protease.

We know just from sequence comparison, one can deduce that the endonuclease within the polymer genes also acted just by sequence comparison, no significant changes compared to recently described active K in the nuclease. We have an intact env gene.

This intact env gene sequence has already been described by Johannes Loewer’s group as an MRA, which also shows that this sequence is actively transcribed. So this is actually an expressed provirus.

We have spliced on the inceptors sides the corresponding position that would allow to splice an

M on A, and what we heard yesterday also, to splice an
additional soft M RNA.

What we see is that this proviral sequence is only defective in the RT domain. It has a single-based permutation within the YXDT motif. So very likely, this highly important catalytic motif is—so only in reverse transcription function this probably missing from that proviral.

Okay. We have here almost intact proviral sequence. But now regarding infectivity, we had that already several times I guess before. We have HERV-encoded retro of particles, several cell lines, even in tissues, the placenta tissue for instance. We find HERV-RNA in these particles.

We have no infectivity so far shown for any of these HERV sequences. We do not really know why. There are several reasons that can be mentioned for the HML-2 family. It has to be reported that the env protein cannot be cleaved into the auto membrane transmembrane domains. It is conceivable that they
are defective genomes that are packaged into these particles, so only if they would be able to get a new cell, they would only deliver defective genomes. It is also not clear whether the receptors, that they were once used by that, retro families are still present and would still be used. So what you should take home I guess is that human endogenous are expressed in several tissues tumor types that are highly up-regulated in certain tumor types. Several HERV families are still able to encode proteins, and among them, the HML-2 family that still encodes all essential proteins. We have almost intact HML-2 provirus within the human genome. Thank you. (Applause.)

CHAIRPERSON RABINOVICH: I think we will hold questions at this point. We are going to take a 10-minute break now. We are going to come back and finish with the last two speakers. I need to figure out how to catch up time,
and yet leave the time for the panel discussions. I
ask you to do two things. Check-out time from the
hotel is 12:00. You should know that. They have
already called in a bunch of the taxis so that if you
need taxi arrangement, please let them know so they
can do that for you. Ten minutes we will start again.

(Whereupon, the foregoing matter went off the
record at 9:55 a.m. and went back on the record at 10:10 a.m.)

CHAIRPERSON RABINOVICH: Is Dr. Broker here? Great.
If you could take a seat please. The next speaker is Dr. Thomas Broker from the University of
Alabama at Birmingham speaking on viral latency-
papilloma virus model.

DR. BROKER: Thank you very much. I would like to deal with two subjects under this topic. The
first is a study of the prevalence of HPV in the
general population, and then following on Dr. Mayer’s
pattern that you just heard, a study of some endogenous sequences in papilloma virus transformed cell lines with some surprising results. We have done some inside 2 hybridization studies of the expression of human papilloma viruses in biopsies from women with HIV/AIDS who were moderately immuno deficient. This is one example, but fairly typical.

What you are seeing is a full thickness of across the cervix. The various probes that we used reveal the expression of one of the major early transcripts of papilloma virus, the E4, E5. You are seeing it here in bright field illumination and dark field, matched pairs, basal layers right there. As I indicated yesterday evening, papilloma transcription is differentiation dependent and occurs typically in the upper half of the skin. E6, E7 messages, the delayed early oncogenes are hard
to see in bright field, but fairly easy to see in dark field. You can see they follow a comparable distribution.

The capsid component, L1 or L2, again, is right at the very top of the last live layers of the epithelium. Also to the point, the vegetative amplification of viral DNA is in the upper half of the epithelium.

Papilloma infections of the genital tract in fact have been designated an official AIDS-defining illness in the syndrome because of the significant upregulation of HPV gene expression in women who have AIDS or other immuno deficiencies. With that knowledge in hand, and pictures like this, we undertook the following study.

We decided to investigate the prevalence of HPB in the population by focusing on immuno-deficient groups. The three that we have chosen so far are: women who are in enstbay renal failure and in
need of a kidney, and most clearly ill; those then who
get a kidney and are pharmacologically immuno-
suppressed beyond their underlying illness; and those
with AIDS.

The strategy that we’re using is an
evolution of the techniques Steve Wolinsky and I
developed really 12 or 13 years ago when we first
proposed the use of degenerate primers for looking at
related genomes. The pair that’s most commonly used
in the papilloma field is our original design called
MY911, but Louise Chao and I moved right next door.
We found that this region is a little too long to use
in form one fixed tissues, and this particular pair
has some wonderful restriction fragment polymorphisms
available that will allow us to do genotyping after
amplification.

So basically we start with the nested PCR
approach, outer primers and inner primers. The
starting material is cervico vaginal lavage, which
harvests cells from throughout the lower genital tract
of the women. We amplify and then we put it through several different assays. Initially, agarose gel electrophoresis to look for a 278-base amplimer.

Secondly, restriction fragment polymorphisms which usually can tell us which genotype is present. But if it’s a pattern that we cannot recognize, we will put it through sequencing. As you are going to see, about half of the fragments that we amplify we need to sequence.

The results of this study, I am going to summarize. It’s absolutely mind-boggling. Seventy-four percent of all women in the AIDS cohort have clearly identifiable HPVs. We have managed to type over 85 percent of these so far. Fifteen percent are still under investigation. In more than half the cases, the individuals yield multiple HPV types.

The study is longitudinal, and has been going on for three-and-a-half years now. Many of the members of the cohort have been sampled two up until
seven different times at six to 12 month intervals.
So that’s our biggest cohort.

These are the renal transplant cohort. We have statistically significant numbers. I would like to point out that in instage renal failure, but no pharmacologic suppression, about 59 percent of those women have detectible HPV. Again, quite a few, a high percentage have multiple infections. This carries over to that portion of this group who go onto actual transplantation.

Some of the remarkable outcomes of the care with which we undertook the genotyping is the following. In the pre-transplant population, the prevalent types are those that are commonly seen in the general population as causing disease, namely HPV-6, 11, and 16. Those types persist in those women who were pharmacologically immuno-suppressed.

We see a scattering of other types, but the common types from prior studies are those that
predominate in the renal transplant cohorts.

In contrast, those women who are in various stages of immuno-deficiency as a result of AIDS, do not show the same genotype profiles. The only member in common is in fact most common of all genital HPVs, HPV-6. What we see instead are niche homologs of the common types. For example, HPV-45, as you are going to see, if a close relative of HPV-18, which is often cited as a common virus. But we don’t see that in the AIDS cohort.

HPV-52 is our most common virus. It is a close homolog of HPV-16, which we don’t see amplifying in this cohort. Most notably are the ones that I indicated by stars, which are a very rare detection within the general population, but in fact are most common viruses in the AIDS cohorts.

In particular, we have identified 13 new HPV types based on less than 75 percent sequence homology to each other or to any other known papilloma
virus. They are all members of what has been
designated group A-3, which appear to be an AIDS-
defining subset of HPVs.

These can be at least considered in the
context of phylogenetic trees based on sequence
alignments in the L1 region. So, for example, HPV-16,
the main cause of cervical cancer in the world, is
seen in the renal cohort, but a very close relative,
52, is seen in AIDS.

Six and 11, that cause benign genital
warts and laryngeal papillomas are here. One of the
main groups coming up in AIDS is this group of cousins
of these guys.

HPV-70 is one of our most common types, as
well as 45. They are in the HPV-18 family, but
represent new members of this niche. The group I just
mentioned, A-3, that is so commonly seen in AIDS,
include our members jyn 2, 3, 4, all the way up to 13,

MM8 and 61, 72, and 83. That cluster seems to be an
AIDS defining group. The other ones that we have seen
abundantly are 51 and 53 in this arm.

Overall, in the Birmingham and generally Alabama population, every virus types seen with the star we have found one up to 23 times, indicating that we have universal presence and also detectibility of all of the known viruses within our immediate population.

While this is up here, I also wanted to point out the very large huge group of epidermal dysplasia formus viruses that other labs have studied. Again, it is a very rare group of illnesses, in fact, only defined a few hundred times in all of medical history in terms of individual patients. However, there is this huge ramification of somewhat related, but clearly distinct genotypes that comprise the family or subgroup of viruses responsible for EV.

It is known that these patients all have particular cell-mediated immune deficiencies. Again, suggesting that particular arms of the immune system
are responsible for either containing or failing to contain different subgroups of the papilloma viruses. As we look at these women over a period of time through these six month or so samples, what we also find, and other labs have exactly the same results, is every time we sample, you may or may not see the type you saw before. It may switch. For instance, we have this patient who had 6 plus 16, and then 11 plus one that was minor and we couldn’t tell, then jyn 2, and then type 40, and then we had a type 53, but the others disappeared. Everyone’s experience in the field has been that the viruses rise above a detectibility threshold, stay there for a while, days or weeks or months, and then fall below detectibility, only to be replaced by a different HPV type. These are not new infections. They are basically cryptic or latent persistent infections that fluctuate in their levels of replication and detectibility. Pretty much anybody
is showing that flexibility.

What I want to state at this moment before showing the correlation with disease may sound controversial, but I will stick by it. We have found a brand new HPV type for every 10 people that we have looked at. Philodelius and Ethel Michelle Diveres and zur Hausen and Shamen in European study of tutanius papilloma viruses have found a new papilloma virus for just about every other person they have looked at when they use the combination of nested PCR and DNA sequencing.

Robbie Burke’s group, Jill Polefski’s group, have very comparable experiences looking at anal papillomas or female genital tract.

It is my contention right now that instead of 80 HPV genotypes or 150 that have been officially named, that there probably are millions of variants, virtually a continuum. We feel that basically everybody has their own personal micro flora, that
these are passively acquired or vertically acquired,
not necessarily sexually, but certainly possibly
sexually, and that they simply are part of the human
condition as are microflora, just as we have
microflora composed of bacteria and many other
viruses, and that they basically are utterly
ubiquitous. I will come back to that point in a
moment.
We did try to correlate the various other
medical parameters in these cohorts, especially the
AIDS cohort, with CD4 count, HIV virus load, other
infecting known STDs like herpes, chlamydia,
trichomonas, so forth. The one correlate that held up
and not surprisingly at all, was that the degree of
pap smear abnormality from normal, abnormal cells of
unknown significance, low grade dysplagias or high
grade dysplagias, is with CD4 count.
The medians, these are all the people who
had multiple infections, a high risk virus type, a low
risk, no virus at all, and had either normal or these

various abnormal pap smears. These bars here are the
median CD4 count in each of these groups.

The one place where we saw active disease,
low and high grade dysplagia, these by median, is when

people fell below the CD4 count of 200 cells per cubic
millimeter.

In summary of that data, we found that

it’s very very possible to have negative pap smears,
but definitely have HPV infections. We feel these are
people who have not yet reactivated long enough to
have resulted in cytologic change as a result of

infection. We have on the other hand, the people with
overt disease by biopsy or by cytology, and the higher
the grade lesion, the more likely it is to see either

single infection or especially multiple virus types
present within that patient at that time.

So the more that we can detect the virus,
that is, the more it has replicated or amplified

throughout the population, the more cells that are
shedding the virus in effect, the more likely we see disease.

So to summarize this part of the talk, I feel that they are virtually ubiquitous. They are typically sub-clinical, persist in or latent infections. There are staggeringly large number of genotypes if we take the care to look. I might say that the reason these are typically not found is that people use generic cross-hybridizing probes or have cut off their probe sets. If you’re not probing for something, you are not going to see it.

Most of the viruses in this number 60, 70, 80 and above, are not even present within the commercial probe sets. So if you aren’t probing, you are not going to see them, and you are going to get lower numbers.

They can be found throughout the genital tract in 60 to 75 percent of the people that we have looked at who are admittedly good yielders, because
they are immuno-compromised, but I think this simply
represents the general infection in the population.

They can be found in oral and esophageal mucosa. Utaneous types persist in hair follicles.
There’s a wonderful study from Amsterdam by Tershaget and Ingebor Boxman. She plucked hair follicles, both eyebrow hairs and pubic hair, and 60 to 70 percent of all people harbored EV viruses or other rare virus types in their hair follicles. No disease, it’s just part of the human condition.

I believe they are vertically transmitted perinatally, mother to baby. Some of them are clearly pre-natal infections. As we know, there’s long-term maintenance that requires viral replication in concert with host replication in the cell cycle.

So what I would like to do now is tell you a little bit about a very unexpected observation we made in Hela cells. This goes back to last night’s talk regarding the structure of the replication
complex of HPVs.

As you know, cyclin E is one of the key checkpoints or entries into S phase. Ectopic expression of cyclin E can speed up entry into S phase, and it can even bypass the need for some of the RB phosphorylation by cyclin D. It’s simply one of the key steps that needs to follow the induction of the DNA replication enzymes.

HPV E7, the viral oncogene that in fact binds RB and can help bypass that step, among the E2F enhenca protein regulated genes is cyclin E itself.

In other words, HPV infection upregulates cyclin E. So we asked whether the induction of cyclin E is essential for the reactivation of unscheduled cellular DNA synthesis in the upper stratum of squamous cepathelium that differentiated caratinocyte. I’ll just summarize that data.

I got you to the point last night where
the E1 diheximer, the double helicase held together by the HSP-70 cochaperone protein, is there. The next thing that loads in the study we did with Theresa Wong at Stanford, is the recruitment of the cellular DNA polymerase, and showed direct interactions between the helicase and the catalytic sub-unit of pol alpha, P-180, as well as its P-70 sub-unit. This was the first indication of what P-70 does in the four sub-unit complex of pol alpha, which includes two primary sub-units. The answer is, it brings the polymerase to the ora itself.

The next thing that comes in is cyclin E, CDK-2 complex, that critical S phase entry point. As a result of that, what happens is upon cyclin E finding an appropriately assembled pre-initiation complex, five target proteins are phosphorylated. They include: the E2 protein, which appears to be displaced by that event; in addition, P-70 helps displace E2. So the loading of this and the phosphorylation kicks this guy out.

Secondly, E1 is phosphorylated. These two
subunits of preliminary salpha that bind directly to E1 are phosphorylated. When all four of those have been successfully modified, the kinase phosphorylates cyclin E itself, which is displaced and degraded by ubiquitination. That enables the pre-initiation complex to convert to the elongation complex.

In studies with Wade Harper and Jien-Ling Ma at Baylor, two things were done. The first is together we found that there’s a cyclin binding motif that the amino terminal have at the E1 protein, which in fact is shared with a number of other things that bind the cyclin E. That motif involves an RXL. That is, an arginine something leucine motif right there. In addition, their candidate phosphorylation cites, the series of serine, serine, serine, and threonine, mutation of any of these, the motif or any of the target phosphorylation cites, diminishes the capacity of cyclin E to convert the
pre-initiation complex to an initiation complex. So
the functional requirement for phosphorylation has
been verified. But keep in mind this location. We’ll
come back to it in a second.

So we assumed that the consequence of
upregulation of cyclin E by E7 gene expression would
identify those cells that are capable of supporting
papilloma replication. To our amazement, we found the
opposition. This is our epithelial raft model. We
have done the same in natural papilloma lesions. Here

we monitored cyclin E expression, over expression in
the tissue. Here’s bromo deoxy uridine incorporation
or PCNA upregulation. These are the match.
What we found is the cells that had high
cyclin E could not replicate. In fact, they are
mutually exclusive with those capable of supporting
DNA synthesis. Conversely, PCNA, which is upregulated
by papilloma 7 and cyclin E do co-localize. But we
see a number of cells where PCNA is present and there
is no cyclin E. So we have a reciprocal pattern to what we expected.

I am just going to very briefly tell you that P-21 cip, one of the inhibitors of cyclin D and cyclin E, is also upregulated by E-7 expression in natural condylomas or in our E-7 expression raft cultures. You can see those signals in the upper strata again. So we have P-21 upregulation, again, in a subset of cells.

When we look in rafts or in natural papillomas, we see that those cells that have high P-21 are mutually exclusive from those capable of supporting either viral or cellular DNA synthesis.

When we did the third pairwise combination and looked at cyclin E and P-21, we found perfect colocalization of those two.

So ironically, the cells that have high cyclin E also have high P-21 and do not support replication. This was really perplexing, except we
did know this inhibited that. But we assumed cyclin E was in the licensing factor for engaging in replication.

So what we came to feel is the following model: that in the course of unscheduled DNA synthesis reactivation, if cyclin E appeared in the appropriate timing or sequence or amount, once a pre-initiation complex formed, you would successfully phosphorylate the target proteins, polymorases and E-1 and E-2 proteins, and successfully engage in elongation.

Conversely, if too much cyclin E appeared and it appeared in an untimely fashion, its inhibitor, P-21, would recognize misassembled complex. They would cross stabilize. They would both pile up to high levels, and those would be defective in engaging in elongation.

Now we put this all together by asking how does this play into the establishment of immortalized and transformed cells and cancers. What Wade Harper
24 had found is that when he did pull-down assays with
25 cyclin E to ask in hela cells what binds to cyclin E,

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1 almost all the things that came down in the assay was
2 that E-1 protein from the resident HPB-18 in the cell
3 lines. Up until that point, people had thought the E-
4 1 gene was deleted from hela. In fact, it’s present.
5 The entire length of the E-1 gene is still present in
6 hela. In fact, is expressed.
7 Now the functional assay that our lab did
8 was that we found that hela cell extracts could not
9 support HPB replication in our cell-free system, that
10 there was a missing factor in hela that the extract
11 needed. We could put 293 cell extracts or any other
12 cell line that we could find, they would easily
13 complement papilloma replication in vitro. But
14 anything from hela eHa caski or any other HPB
15 transformed cell line could not support it.
16 The upshot of the whole thing is that
17 every papilloma transformed cell that we studied
expressed a full length E-1 transcript, but in all cases, the transcript had an either frame shift or a stop code on partway through the gene or miss sensed mutations in this vicinity, so that in tact E-1 could not be made. But in all cases, it made the RXL portion that interacts with cyclin E.

So we added a little cyclin E back to hela cell extracts and immediately restored full complementability to those extracts, establishing that the missing thing in hela cells was cyclin E.

So our conclusion is the following. I think we feel very confident about it. In the process of immortalizing cells, either natural cancers or attempts at making cell lines, substrates in effect, it’s good to have all the upregulation of DNA polymerase, topasomerases, PCNA and so forth, that help rapid cell cyclin. But the one thing that E-7 upregulates you don’t want, is cyclin E.

So what these natural experiments did, is
figured out a way to sequester part, but not all, of the cyclin E by putting in retaining fragments of E-1, capable of mopping up that one product that’s upregulated that you don’t want to have. That is, cyclin E.

Brian Van Tine, last night, also indicated there’s evidence of some antisense in other papilloma lines like cHa caski, which would again, modulate the amount of E-1 that you could translate from the messages that are clearly there.

Together we believe that to establish these cell lines, whether you make them in the lab or whether nature has made them for you through cancer, you need to diminish the amount of cyclin E to achieve an equilibrium where you have enough cyclin E to support cellular replication, but not too much so that it’s an unsuccessful high level. Thank you very much.

(Applause.)

Oh, I did want to acknowledge—could I
have the slides for one second—a very, very large
number of collaborators. I’ll leave them up here.
But we’re very grateful for our own students and
collaborators at UAB, Jeff Engler, Doug Seer, Sean Van
tine, Kim Towns. At Baylor, Wade Harper’s lab, UNC,
Jack Griffith’s lab, who did the things with the HSP-
40, and at Stanford, Theresa Wong, and our
collaborators at the Free University, who did a lot of
work on some transcriptional control that we
collaborated on for several years. Thanks.

CHAIRPERSON RABINOVICh: Thank you. Are
there any questions?
DR. FRIED: How could you be so sure that
with the evolution of the HPVs, are not due to new
infections, but to pre-existing sequences to cover?
DR. BROKER: I think one is the repetity -
- 

DR. FRIED: Mike Fried.
DR. BROKER: With which these viruses are
appearing, especially in women who at least declare
they are not engaged in much, if any, sex. A number

of the epidemiologic studies have traced either the

frequency of recent sexual activity. Everyone who has

looked feels its an emergence of pre-existing

subclinical infections. There is some evidence in new

infection, but an awful lot of it simply appears.

Women in that stage renal failure, for

every example, who are 38 years old and married basically,

are not suddenly acquiring new infections.

CHAIRPERSON RABINOVICH: Back microphone,

please. Why don’t you come up to the front.

DR. RUSSO: Hi. Carlo Russo, from Merck.

Very interesting talk.

I have a couple of questions. One is, how

can you be sure that what you are sampling by PCR is

really an infective virus, it’s not just a transient

presence with a virus due to the fact that you can’t

control sexual behavior, and perhaps the woman has

just been exposed to a virus?
DR. BROKER: Well, I think one way is that in a very high percentage of the people, there are clear dysplasia, low and high grade. All the women who have any degree of dysplasia are also biopsied, and the inside 2 hybridization, as you can see, is showing clear effects in the tissue.

So I don’t think there’s surface adventitious contaminants. These are within, at least a fair number, if not all, are within the cells, and causing various degrees of actual overt illness.

DR. RUSSO: I may have missed the data.

Did you show the types that are associated with high grade lesions, the HPV types? I didn’t see on the table.

DR. BROKER: Yes. Well basically, in this immense spectrum of what’s now 37 different viruses that we found, those that are most typically associated with low and high grade dysplasia, the actual diseases, are the higher risk types.
DR. RUSSO: So you are not suggesting that
if you want to prevent cervical cancer, we should
focus on different types of the one already
identified?

DR. BROKER: Well the real problematic
thing for any clinical management, either vaccination
programs or small molecule drugs, is this absolutely
exploding number of virus types.

The one thing that I think is going to --

and I commented a day or two ago that in the U.S.
alone today, there are over 250 to 300,000 people
immuno-suppressed just due to organ transplants,

steroid use, or bone marrow transplants or AIDS. So

there is an immense reservoir of particularly high
risk patients.

Nonetheless, most of the diseases are
still being caused by a handful of viruses like 16,

18, 52. So I think, at least the ones we have to
worry about today, are still manageable in number.
AUDIENCE MEMBER: I would like to ask a little bit about cell substrates. Considering that hela has multiple HPV integrants, I guess, are any of those infectious?

DR. BROKER: No.

AUDIENCE MEMBER: Can you get them back and make them infectious?

DR. BROKER: No.

AUDIENCE MEMBER: What are they missing?

DR. BROKER: They are all truncated within E-2, at best. Although there are 30 to 50 copies, depending on the hela sub-1. All the integrated copies are truncated.

AUDIENCE MEMBER: Would that DNA be transforming, even not infectious?

DR. BROKER: They do contain the E-6 and E-7 genes. Expression of those genes, as shown by studies primarily in zur Hausen’s lab, must be maintained or you no longer can cycle hela cells.
That is antisense to E-6 and E-7 in hela makes them not cycle any more. So the driving force of hela is the overt expression of E-7.

AUDIENCE MEMBER: Would hela cells qualify as an example of a cell that should never be used to make a vaccine?

DR. BROKER: I don’t know that I would go that far. It’s obviously wonderful as a producer of all sorts of biomedical products.

DR. MURPHY: I actually wanted to ask you the same question, but I would phrase it in a different way.

(Laughter.)

DR. MURPHY: This is Murphy from NIAID.

That is, do you see any reason why, you know, having an intimate knowledge of hela cells and human papilloma viruses, that hela cells should not be used as a substrate for making live attenuated virus vaccines?

DR. BROKER: I don’t know of any evidence
of these genes being transduced out or in any way posing a risk. I was going to save it for the panel, but it occurred to me last night, I had challenged one of last night’s speakers about the use of psorilins as a cross-linking agent. It gives me great concern that it is a known carcinogen. However, Brian and I were talking. Brian van Tine and I were talking last night, and he reminded me that there are biotinylated psorilins. So for all the debates regarding how to remove contaminating DNA, one strategy in principle is throw in a biotinylated psorilin, cross link it, and pass the whole thing over avidin magnetic beads or batch subtraction of the DNA. So in fact, that strategy may actually help you deplete adventitious contaminants very, very readily. So it is an alternative at least.

AUDIENCE MEMBER: Can I just ask you about hela again? We learned last night that not every HPV,
if there’s 30 to 50 copies, are not all active, I mean
in caski only one was active.

DR. BROKER: Yes.

AUDIENCE MEMBER: What is the state of

tella? Are they --

DR. BROKER: Very, very few are active.

We and Wade Harper’s lab are both sequencing all the

transcripts. This actually was done by Elizabeth

Schwartz and others in zur Hausen’s lab in 1985, and

a variety of groups since then in Japan and elsewhere

have looked at the expression loci in copies in tella.

There appear to be three or four different

transcripts made from different positional integrants,

but the majority are silent. A few of them are

active. But so far, all the ones that are active have

truncated E-1s. They have the cyclin E binding motif,

but they don’t have their normal carboxy terminus.

CHAIRPERSON RABINOVI: Thank you very
Let’s go on to Dr. Cashman. Thank you for being so patient. Transmissible spongiform encephalopathies: vaccine issues.

DR. CASHMAN: It worked. My friendly A/V guy explained how to do this.

I am Neil Cashman. I am predominantly at the Center for Research and Neuro Degenerative Diseases at the University of Toronto. I have a special interest and a long-term research effort in the expression and function of the normal cellular isoform of the prion protein.

I am also obliged to mention that I am the chief scientific officer one day a week of a little biotechnology company in Montreal called Caprion.

I want to spend a few minutes talking about prions and prion disease. We had a speaker yesterday who said “and now for something completely different.” Well, how does a genomeless infectious
agent grab you?

Creuzfeldt-Jakob disease is the most common human prion disease that we run into. I do want to spend a few minutes talking about this so that we are all on the same page with regard to public health risks. Creuzfeldt-Jakob disease or CJD is basically a disease you wouldn’t want to wish on your worst enemy. It is a completely untreatable uniformly fatal disease resulting in death within six to nine months of presentation. Survival over a year is recorded, but it is not very frequent.

The presentation is usually that of a kind of Alzheimers-like syndrome, with problems in memory and intellectual function, but it can also present as a disorder of gait and balance as well. Most people have mild clonus, which is twitching of the muscles, sufficiently forceful to move a joint. Other features of the neuro-degenerative syndrome are reminiscent of other neuro-degenerative diseases like Lou Gehrig’s disease and Parkinson’s disease. Basically it’s like
having every neuro-degenerative disease at once,
telescoped into an unmercifully short period of
decline.

Fortunately, it is rare. Sporadic

Creuzfeldt-Jakob disease occurs at about one per
million population per year. Also, somewhat
fortunately, it’s not a disease of children. The
average incidence of CJD is in the 60s.

There are three recognized forms of CJD.
The most common being sporadic. This is a spontaneous
onset of CJD in an individual for which we have no
cue why they have developed it. There are familial
variants, which seems to be passed as an autosomal
dominant in families. That constitutes about 15
percent of the cases of human prion disease that
occur. There are iatrogenic prion diseases, which are
caused essentially by treatments and surgeries, well-
meaning, but nonetheless transmitting the disease.

Of course the transmissible spongiform
encephalopathy that even my kids know is bovine spongiform encephalopathy, or so-called mad cow disease. Since the early 1980s, this disease has affected about 200,000 cattle in the U.K. and Republic of Ireland, and a few hundred across continental Europe. About 2 million cattle have been killed in an attempt to stem the epidemic. This culling, as well as change in policies, such as feeding ruminant to ruminant—we turned cattle into neo-cannibals—is resulting in a rapid decline of new cases, predictions being that the epidemic in cattle may be essentially stamped out in the early part of the new millennium. I won’t even say the new century. I’ll say the new millennium.

Unfortunately, this disease is unique, unlike every other known naturally occurring prion disease. It doesn’t seem to obey species barriers, or at least obeys them to a much lesser degree. There is an outbreak of feline spongiform encephalopathy in
house cats. There is spongiform encephalopathy in zoo
animals, including primates. The primates that we are
most concerned about are also vulnerable to this
disease.
To date, 44 people have developed a new
variant of Creutzfeldt-Jakob disease, which is
clinically and pathologically distinct from classical
CJD. The statisticians predict there will be
somewhere between a few hundred and maybe 80,000
cases. This does not include the chicken little
predictions of the extent of the epidemic.
The disease unfortunately seems to strike
the young. There have been teenagers involved. It is
a relatively slower progression than classical CJD.
There are clinical features that are distinctive, but
I won’t bore you with them this talk. The pathology
is also absolutely distinct, including a preter
natural accumulation of PrP Sc, which is this abnormal
amyloid protein that’s been linked to infectivity.
This occurs both in the brain and in peripheral lymphoid tissues.

Well, before we leave the clinical stuff about CJD and prion disease, I want to kind of set the stage with a sobering statistic, which is there is iatrogenic transmission of this group of diseases. Considering the penetrants and the young age of vaccinees, this is a scary possibility. This would dwarf every other iatrogenic transmission known to date.

In humans, basically a few hundred cases have been attributed to iatrogenic transmission, from hormones extracted from cadaver pituitaries, from dura mater transplantation, which is the tough lining of the brain. But incredibly, the largest iatrogenic transmission known to date, also the first documented, was that in passage with a vaccine, which was a vaccine for looping ill of sheep. Formal and inactivated brain preparations passed sheep scrapie to about 1,000 sheep. So hopefully this will not be a
pattern with human vaccines.

Well here is the prion hypothesis. This has gone from being an object of ridicule to the middle of the road interpretation of prion infectivity. It has been sanctioned by the Nobel Prize committee, garnering the prize for Stanley Prussiner, the investigator whose ferocious work with this group of disorders and with this agent has given him, in my opinion, a well-deserved Nobel Prize.

The basic tenants of the prion hypothesis are that there’s a normal cellular protein, which is called PrPC, which has been cloned and recognized. It’s expressed by just about every organism down to drosophila. It is a very old gene. It’s incredibly well conserved in evolution. It is predominantly alpha helical in secondary structure.

Now this normal cellular protein can adopt an alternate confirmation, which is rich in beta sheet structure. When this protein is in this alternate
confirmation, it acquires many unique physical chemical properties. It becomes partially protease resistant. It tends to aggregate. It’s very poorly soluble. Plus, it then seems to act as a catalyst for recruiting more conformational copies of itself. Now whether this occurs by a kind of enzymatic confirmase activity or whether this is kind of a biological crystallization phenomenon is being actively investigated. But it is clear that this abnormal confirmation isoform of the protein, called PrP Sc for scrapie, is capable of recruiting more confirmational copies of itself from the normal cellular isoform. So onto vaccines. There are some concerns about vaccines. I will mention three areas that need to be considered. I will dwell most of the time on cell substrates, which is nice of me considering this is a cell substrate meeting. I will also talk briefly about potential prion infectivity coming over in media.
supplements for those cells, and in excipients, which
are compounds used to stabilize vaccines in their
final formulation.

In this case, luck seems to be at least
partially on our side, because it’s not easy to infect
cells in vitro. It is possible to infect primary
neuro cultures. It is even possible to infect
neuronal cell lines neural blastoma. But there is not
much infectivity, and basically each successful
infection of a cell line is worth a publication or 10.

This may be due to the fact that cell
lines have very little PrPC, which is the precursor
for PrP Sc. The conversion of the protein from PrPC
to PrP Sc forms occurs at the surface or a post-
surface compartment. So in general, cell surface
abundance of the protein correlates with infectivity.

Most cell lines in my own laboratory, including hela,
express no more than one-tenth of the amount of cell
surface PrPC that a primary neuron does.
It has also been thought that the very act of cell division itself can kind of sterilize a culture because cell division can outpace the relatively slow conversion and processing of PrP Sc. So if you have a couple units of infectivity, they get progressively diluted by having huge numbers of cells that bear no infectivity. Finally, those cells may die, the infected cells.

There is also a poorly quantifiable role for cell biology, which I put in quotation marks here. Things that we really cannot quantify at this point, like proper trafficking, post-translational modifications of PrPC that are important in conversion, and even sub-cellular distribution. The protein seems to accumulate in this glycosal phosphatindinol rafs at the cell surface. Some cell lines don’t seem to support these sort of rafs.

Now to make some kind of estimate about the spontaneous development of prion infectivity in a
cell culture, especially a vaccine cell culture that may have hundreds of trillions of cells, I am going to back up and try to explore some assumptions about the spontaneous development of prion disease in humans, which is the species for which we have the best numbers.

According to the prion hypothesis, an occasional accidental mis-folding of PrPC to PrP Sc is what triggers the recruitment process which proceeds on an exponential basis. Each molecule that’s converted converts to more, da, da, da, da, da, da, which happens on a post-translational level. No genome involved. “Look, ma, no genome.”

But sporadic disease in humans is incredibly rare, one per million people per year.

Humans have something on the order of 100 billion neurons. So one can make the kind of interesting calculation that a productive infection arising from a single neuron, you need about 10 to the 17th neurons, 100 million neurons across a million people
in order to develop spontaneous scrapie.

But of course that’s not the only way one could potentially develop spontaneous CJD. Discovered by familial CJD and familial prion diseases, there are mutations within the open reading frame of the prion protein that apparently predispose to this accidental misfolding, such that somebody with a prion protein mutation that actually results in an amino acid substitution is basically guaranteed of developing the disease over the course of a lifetime if he or she lives long enough.

So could this occur in vitro? Could certain cells in vitro acquire a somatic mutation which is then propagated to infect an entire culture, again, on a post-translational level? Well, let’s run some numbers on this one. The mutation rate in man is about one per billion basepairs per year. I thank Dr. Kazazian for yesterday for pointing me to this reference. Thank
The prion protein open reading frame is really less than 1,000 basepairs. It’s a relatively small protein that’s all contained in one exon. This gives rise to a kind of pseudo calculation that a cell can develop a mutant prion protein gene, a cell in vivo, and a human can develop a mutant prion protein gene in about one out of a million cells per year, if you take one out of a billion and multiply it by 1,000, that’s one out of a million.

This, just as an aside, this gives a rise to a kind of startling calculation that all of us in the audience are generating about 100,000 prion mutants in our brain per year.

However, there must be a safety factor here because the rate of prion disease arising from somatic mutation cannot exceed the calculation of prion disease arising from individual neurons that we just went through in the last slide, which is 10 to
the 17th neurons per year.

So incredibly, somatic mutation is a seriously flawed non-efficient process for producing prion disease. Something on the order of one out of 100 billion mutations are productive of infection. This may give us some comfort when we turn to the in vitro scenario.

So let’s talk about cell substrates. Is it possible that spontaneous prion infectivity could arise de novo in a culture? I told you that substrate cells have usually less than one-tenth PrPC than neurons. So if we run these calculations, by misfolding one substrate cell per 10 to the 18th years, and I think that is a quadrillion or something like that, it’s certainly comfortably larger than the projected age of the universe.

Substrate cells, however, are less genomically stable than primary neurons. If one says
that there’s say 1,000-fold greater rate instead of
one out of a billion basepairs, one out of a million
basepairs can be mutated per year in a substrate cell,
this gives rise to a calculation suggesting that you
need 10 to the 14\textsuperscript{th} substrate cells per year in order
to have one productive, i.e. spontaneous infectivity
arising in a culture.

Now this number looks incredibly large,
but when we think about the numbers we heard last
night about the production of polio virus from vero
cells, according to my calculations, 300 million cells
are used per year to generate all those vaccine lots.
Ten to the 14\textsuperscript{th} is only 100 -- sorry, 300 trillion,
and 10 to the 14\textsuperscript{th} is only 100 trillion. Did I get
that right? Please forgive me and correct me if I
didn’t get it right.

So perhaps it is possible, considering the
everous scale of substrate cell culture, that prion
infectivity could arise through somatic mutation in a
substrate cell, and could contaminate a vaccine
destined for human beings.
Well, there are some things to talk about with this model. If this is so, how come we haven’t seen any vaccine transmissions yet? One of the factors is that very few human cell line vaccines have gone into humans, certainly not on a scale of vero cells or primary cells that are used for culture. Another unsettling thing is that if indeed there is somatic mutation in a culture of human substrate cells, would we ever detect it? We are talking about something that would occur in one out of a million cells or even one out of a thousand cells, would ever be able to find by PCR or SSEP or anything you could think of, a mutation at this level. So aside from substrate cells, I did want to touch upon a few potential sources of infectivity. The media coming in contact with substrate cells are potentially carrying prion infectivity. Bovine serum, fetal calf serum, and newborn calf serum is used as a
supplement for proteins and growth factors and
hormones for most cell lines. Some cell lines are
also supported by human serum albumin. I am not aware
of a vaccine cell line that’s supported in this
manner, but many recombinant proteins are supported
with human serum albumin.

There’s also potential prion infectivity
in excipients, this last compound that’s added to the
preparation to keep it stable before use. Many
childhood vaccines are stabilized with pig skin
gelatin. Pigs don’t seem to be a species which
spontaneously develop prion disease or a species that
can catch prion disease via the oral route, although
deliberate intracranial injection of BSE infectivity
can produce a prion disorder.

Human serum albumin is also an excipient
in measles, mumps, rubella, and rabies vaccines. I
would like to spend just a few seconds talking about
human plasma proteins before I close, and give my
final advice, such as it is.

Human serum albumin of course comes from humans. Of course it’s a plasma fractionation product. There has been a great deal of work trying to identify potential risks of transmission of CJD from human to human through blood or blood products.

Suffice it to say that population studies, case control studies, and cohort studies have proven universally negative. There is no documented incidence of human CJD, classical CJD being passed through blood or blood products.

There are of course case reports of people getting a transfusion and developing CJD, but one should not expect that transfusion or administration of a blood product is protective against CJD. The incidence of CJD in the transfused or treated population is the same as in the non-transfused or treated population.

However, we’re in more difficult territory
with variant CJD. This is, I told you, an unusual
agent. It seems to cross species lines with impunity.

There are other features that are quite scary with
regard to human blood and human plasma products,
including albumin that might be used as an excipient.

The agent starts of course in the
periphery through oral exposure, suggesting a
prionemia. There is a huge accumulation of PrP Sc,
our only surrogate for infectivity, our only
biochemical surrogate for infectivity, in not only
brain, but in lymphoid tissue.

The agent itself has odd properties. It
is stable across species. It may in fact be
specialized or selected. I realize that these terms
are not often applied to a protein. I'm borrowing
terms from agents that contain a genome. It suggests
that this agent may be more virulent, especially with
regard to peripheral exposure. In other words, one
unit of classical CJD infectivity will not cause
disease when injected intramuscularly. One unit of
Another thing that has been noted is the young age of onset of variant CJD. This has been attributed to kids eating hamburgers and all kinds of weird meat products. But it could also be attributed to host factors which would promote infectivity in the young. Since vaccinees are usually young, we have to take this in mind.

But our greatest risk factor here is that this is a new disease and we have no data. So how do we minimize the risks from vaccine transmission? Basically there’s three ways that I can think of. I’m sure that other people can think of more. It is important to add prion validation to the list of agents and microbes which are tested for in vaccine lots.

This could be done two ways. The biochemical marker of infectivity is PrP Sc. This
technology is in evolution. It appears that capillary

electrophoresis, some types of optimized immuno

 blotting, and even ELISAs are reaching the point where
 one unit of infectivity will be detectible.

Another important way of assay for

infectivity is called the bio assay in the field, in
which selected samples are injected into a species
which is capable of supporting that infectivity. That

would be non-human primates, and again, a technology

in evolution, transgenics engineered to express human
and perhaps bovine PrP.

There is also the possibility of trying to
prophylax cultures, substrate cultures with chemical
agents. This is also in evolution, but the classic
molecule in this regard is congo red, which not only
seems to bind to PrP Sc, but seems to dissolve

infectivity in vitro. New discoveries out of Byron
Kohe’s lab that tetrapyrrole, including porphyrins and
phthalocyanines, can block infectivity. Perhaps some
of these compounds can be utilized at appropriate
cconcentrations to use as a kind of antibiotic for
substrate cultures.

Finally, the slam dunk in this area would be to develop a cell line that lacks a prion protein
gene. The prion infectivity, whatever the hell it is,
seems to be absolutely dependent on the presence and
expression of PrPC. So if one were able to ablate the
prion gene out of a substrate cell, that didn’t come
with 300 other bad pathogens, this may be a strategy
of obviating any prion infectivity in vaccines.

So I would like to summarize by saying that it is possible, although not favorable, for
substrate cells to be infected with prions. It is
possible, considering the huge bulk of cells that are
cultured, 300 million a year for one vaccine, that
prion infectivity could potentially emerge by
misfolding and/or somatic mutation in vitro.

I will note that prion components and
excipients may contain prion infectivity. Although this is an old story with regard to classical CJD, we don’t have the information for the BSC variant CJD agent. We should worry, at least for the time being. The remedies for this are selective sourcing, avoid animals and people that could potentially be brewing prion infection, biological manipulation in vitro, including anti-prion agents, and maybe ablating the prion gene, and then validate, validate. Prion infectivity should be added to the list of infectivities that are excluded in vaccine lots. I thank you for your attention, and I would be glad to answer any questions. (Applause.)

DR. KRAUSE: Phil Krause, FDA. In keeping with the idea that one presumably wants to find cell substrates which carry the least risk, I guess if one presumes that tumor cells have a greater risk of genomic instability than non-tumor cells, are you then
implying that there’s a greater sort of spontaneous
mutation than prion risk from tumor cells than for
instance primary or diploid cells?

DR. CASHMAN: That’s a good question. I

guess this is basically not quantifiable. If one

takes a rock solid cell that enjoys all kinds of DNA
repair mechanisms then yes, that is less likely to
give rise to the mutation in the prion protein gene.

One area which should be investigated, I’m
realizing from this meeting, is to take some cell
lines and look at 1,000 clones a piece and see if any
of the prion copies have acquired mutations. So this

would be a piece of data that we could use to actually
discuss this issue. Right now, I don’t have any.

AUDIENCE MEMBER: Along the same lines, I
guess the implication would be that if somebody wanted
to produce a vaccine in cells that are derived from
neurons, given the fact that they are making a lot
more of this, you have the potential for greater RNA
polymerase mutation rates, and perhaps also greater
risks?

DR. CASHMAN: Well, neurons are the best
factoring for making infectivity, both in vivo and in
vitro. Part of that is due to high levels prion
protein. Part of it is due to factors that haven’t
been identified as of yet, prion receptor, trafficking
of prion protein. So I guess one would want to stay
away from neurons unless they came from an animal or
human that had the prion gene locked out or not
transcribed or translated.

CHAIRPERSON RABINOVICH: Dr. Egan?
DR. EGAN: As Dr. Minor mentioned before,
Japanese encephalitis virus is made in mouse brains.

Can the PrP SC of a mouse catalyze the conversion of
human PrPC to PrP Sc?

DR. CASHMAN: That is an extremely good
question. I neglected to mention in my talk, my
oversight, that there is a very prominent species
barrier between most prion agents, something like one
to a thousand, one to a million, even higher for

generating infectivity, especially between widely
differing species.
Now as we move to human cell substrates,
we will no longer be protected by the species barrier.

Even non-human primates have a sequence similarity to
convert human PrP and vice versa.
So yes, I think that answers it. The

exception to that is of course this new variant agent
which we’re all frightened about because it doesn’t
seem to obey species barrier.

DR. ONIONS: David Onions. Could I just

ask the converse of the question that you posed for
vaccine substrates, which is I think one that has been
discussed. That is, the idea of knocking out the PrP
gene. We know that PrP mice are viable, so it looks

like perhaps an interesting way to go.
But can you also engineer cells over
expressing normal PrP and use those as substrates for

infectivity? You mentioned that one of the problems

was the low level of PrPC in most of the cell lines

you have used. Can you not over-engineer cells so

that they become susceptible?

DR. CASHMAN: The only—that’s never

been done. The only data that really pertains is

transgenic mice. In fact, if you have knocked out the

prion gene, you are absolutely resistant. If you have

one normal copy, you have disease with a longer

incubation and a shorter rate of—or a longer rate

of progression. If you have two normal copies, you

have disease at the normal time. If you have

transgenic 10, 20, 100 copies, then the disease

presents at an earlier age and is more rapidly

progressive.

As far as I know, nobody has moved that

observation to an in vitro paradigm.

DR. LOEWER: Johannes Loewer, Frankfurt.
I would like to challenge, to some extent, your calculation on the risk of mutations, because they do not take into account the similar biologies of prion protein that are secreted from the cell, as they can spread to other cells. That there’s really a short time induction of new PrP Sc. For example, at least to my knowledge, if you infect so to say cell cultures who use prion via PrP, you get another multiplication of infectivity. It stays more or less. If it disappears, it may be more or less there’s no real spread.

The question I have is, you are asking for validation studies. What would be the material you would recommend to spike? Should we spike with scrapie brain material? Is this relevant for purification in these cases? What would be the ideal spike?

DR. CASHMAN: I would very much appreciate some challenge from my numbers. They are new numbers, so don’t hold back.
The question of spiking, there have been experiments performed at least with a purification of albumin and other plasma proteins. Paul Brown and Robert Roewer published an article last fall in Transfusion detailing the amount of infectivity if you start from a high amount in whole blood, what you end up with in albumin. In fact, there’s at least a 10 to the 6th loss of prion infectivity if one follows the normal protocols for purification of albumin.

The question is whether a single infectious particle is there. The other question is what happens if this single infectious particle is variant CJD. If the single particle is classical CJD, nothing would happen. The disease would not take hold. We don’t have enough information about the virulence of variant CJD to be able to answer that question with any confidence.

Did that answer your question? I have a feeling it didn’t.
DR. FRIED: Mike Fried. I think your numbers also don’t take into account modifier genes, which could be just random mutation.

For instance, I understand that all the people with the new variant CJD have a polymorphism of one type in the PrP, the protein. Is that not true?

DR. CASHMAN: In fact, that is partially right. There is a normal polymorphism in the prion protein open reading frame at codon 117. One can either have a valine or a methionine. To date,

everyone—well, there’s a nice distribution in the normal population, about 50 percent are heterozygotes and about 25 percent are met met, and 25 percent are val val.

In a new variant CJD, everyone who has contracted the disease to date has been met met. But that in fact may be due to the fact that the met mets are more susceptible to the agent. It may not be due
to the fact that val vals or heterozygotes are resistant.

DR. FRIED: Sure. I am just saying that that goes into your numbers, whether there’s modifications.

DR. CASHMAN: The point of modifier genes is a very important point. I tried to -- 129, I’m sorry. That’s David Asher who was keeping me honest in more ways than one. It’s the polymorphism is at codon 129.

Now what was I going to say? I was going to say something.

CHAIRPERSON RABINOVICH: Modifier genes.

DR. CASHMAN: Modifier genes, yes.

Modifier genes have of course been proposed from animal studies of infectibility and experimental scrapie. Dr. Prussiner has hypothesized a protein X, which may be a receptor or may be a chaperon that somehow modifies susceptibility of an animal to prion
diseases. There is also a protein Y that Dr. Prussiner has hypothesized.

I agree with you from the bottom of my heart that there will be modifier genes affecting susceptibility to prion diseases and the propagation of prion infectivity in vitro. But we don’t know what they are yet. At the crude operational level of being able to infect cells, yes. We can infect cells in vitro. So at least some of those modifiers have to be there. Did that make sense?

CHAIRPERSON RABINOVICH: Dr. Hayflick, final question.

DR. HAYFLICK: Hayflick, UCSF. I was intrigued by your observation that the species barrier for prion transmission is less, is reduced between non-human primates and humans, which would raise some additional concern about the use of primary tissue, and particular, primary monkey kidney tissue for the production of human virus vaccines, because contrary to popular belief, that tissue and any primary tissue
does not contain—and I’ll use primary monkey kidney as an example, only cells that are derived from a particular part of the kidney.

A primary monkey kidney culture consists of an enormous variety of differentiated cell types that compose the vascular system and neurons. So that monkey neurons do play a part in the production of polio virus, for example, derived from monkey kidney. So that I think that it’s important to mention that neurons are not only a part of brain tissue in considering various cell substrates.

Also I was wondering whether there’s some reason why you omitted the mention of trypsin as potential source in your discussion of substrates or media supplements for prion transmission. I didn’t see trypsin as a component. Was there some reason for that omission?

DR. CASHMAN: Just blanking out. So thank you for adding that to the list.
DR. HAYFLICK: The final question I have is I may have misunderstood your slide in which you were addressing the question of utilization of human cell line, that a human cell line had not been used for the production of enormous numbers of doses of vaccine, for example, in order to support one of your contentions.

Did you mean it in that respect, a human cell line that is defined to be immortal and transformed? Or did you mean any human cell population?

DR. CASHMAN: Perhaps you could educate me. Has any human immortal or neoplastic cell line been used to develop large penetrants, large population vaccines?

DR. HAYFLICK: No. Not as you have defined that cell population. I was interested particularly in normal human cell populations, which have been used for the production of up to a billion,
with a B, doses of virus vaccine. But you are not

including in that?

DR. CASHMAN: Say it again. I’m sorry.

DR. HAYFLICK: Normal human cell

populations have been used for the production of about

three-quarters of a billion doses of human virus

vaccine world wide, but these are not continuously

propagable abnormal heteroploid cell populations.

These are normal finite lifetime cell populations.

DR. CASHMAN: So these are cell strains?

DR. HAYFLICK: Yes, as I defined them. I

realize there’s a problem in understanding these

terms.

DR. CASHMAN: Which vaccines?

DR. HAYFLICK: Virtually all pediatric

vaccines, polio, rubella, mumps, measles, rabies,

adenovirus, some rhinovirus vaccines, are all produced

on a semi-continuous human diploid cell strain like

WI38 or MRC5.
DR. CASHMAN: Thank you.

CHAIRPERSON RABINOVICH: I think you better clarify.

DR. LEWIS: Yes. To my knowledge, there are really no nerve cells in the kidney. The nerve cells lie on the spinal cord and porosises go down there. I don’t believe there are any nerve cells in the kidney. Even if they are, once the nerve cell is differentiated, they basically do not grow on tissue culture. I think that needs to be corrected.

CHAIRPERSON RABINOVICH: Okay. I would like to move rapidly to bring the panel members up to the podium, and to invite Dr. Onions to come over and run things.

DR. ONIONS: I notice that we now have actually 45 seconds for discussion according to the program.

(Laughter.)

Brilliant as this panel are, I don’t think they could do that. So could I have some guidance on
when you would like to close this panel session?

Perhaps somebody could give me some guidance.

CHAIRPERSON RABINOVICH: Forty five minutes.

DR. ONIONS: Forty five minutes. Thank you very much.

Okay. We were charged in this panel to answer a number of questions. I'll come to those and try and go through and cover the areas that the panel will discuss. I would be very grateful for as much participation from the audience as possible.

I thought it was just useful to pick up two strands that I think came out of some of the comments yesterday. One I think that’s important to make, and that is that vaccine production is a very pragmatic process, and that once there have been lots of theoretical objections to particular cell substrates, particularly autogenic cell substrates, there are very practical reasons for the use of cell
substrates that might be immortalized or neoplastic from a new generation of vaccines. I don’t think we should lose sight of that. There are very practical reasons in scale up and use that I think we should bear in mind to produce therapeutic vaccines.

The second concern was that came out, and perhaps didn’t get enough airing as it should have done, and that is there clearly is a trend in society at the moment about concern in safety of vaccines. That perhaps therefore focuses particularly on the item we are going to discuss today, which is the possibility of adventitious agents.

The question that the FDA asked us to evaluate, or at least one of the first questions, and they would like the panel to take a look at is do neoplastic cells represent the greater equivalent or lesser risk for the presence of adventitious agents than primary cells, diploid cells, or non chunogenic continuous cell lines.
I am not sure, given my previous comments, whether I think necessarily that you can answer that in a simple sense, but does anyone in the panel want to sort of pick that one up first of all? What I could perhaps do is to prompt people, is perhaps to put up those which is just my suggestion, of some of the factors that might influence the risk of adventitious agent testing in a variety of cell substrates.

DR. MINOR: I am personally convinced that they are going to be better than primary cultures. I am not sure whether they will be necessarily better than human diploid cells or anything else if we even get a decent banking system going. It seems to me that when you get to that kind of stage, it’s the concern about how you find what’s in there rather than anything else. I think the actual extent to which you can characterize them is clearly to me, it’s very similar.
DR. ONIONS: That would be a generally universal statement that primary cells are likely to be more difficult to characterize and therefore, if you can use a cell line, that is probably the way to go.

But I think it’s also worth making a countervailing point that there are still vaccine strains that are very successfully produced in primary cells. There are others that have been passed in primary cells and therefore change them into a cell substrate, the genetic stability of them. So there are nevertheless countervailing arguments.

I think the statement is correct. That is, adventitious agent testing is clearly going to be more in the region --

DR. ROBERTSON: Another point which one could consider. Where neoplastic cells might be considered more susceptible than primary cell cultures, in that the primary cells are derived
specifically for vaccine production. Whereas the
neoplastic cells have probably been kicking around at
least one, if not several laboratories, before being
put into use as manufacturing of vaccines.
Because of that, they may well have picked
up something that you don’t want to be there. Nothing
to do with the cell type or the origin of the cell.
So a virus of some different species all together
which you really have got to check for.
So if you are actually setting up a cell
bank of a neoplastic cell, you shouldn’t just be
considering species of origin of that particular cell,
whether it’s porcine, human, murine whatever. You
have really got to consider any virus under the sun.
We know there have been instances of this happening.

This morning there was the comment about
I think it was a human endogenous retrovirus which was
actually of murine origin, been picked up from passage
somewhere.

DR. ONIONS: There is a good example in a
commercial product. That of course was the Glaxo
Wellcome’s novalma cell line which was used to produce
interferon, which in fact contained SNRV, and probably
picked up SNRV in George Kahn’s lab at some point
during its history, I think was the general consensus.

But clearly that was unknown and the whole system was
used in the production for several years before it was
realized that perhaps this was contaminated by an
adventitious agent that you just would not expect in
this cell line. So I think that is a very good point.

There are issues about tumor cells. One
of the things that occurs to me is that—actually it
does concern me quite a lot about adventitious agent
testing. That is that it is rather traditional in
character still. It is not very directed in terms of
its specificity in looking for certain viruses. That
is changing I think, but I think until recently that
has been the case.

For instance, we have known that in
certain tumor cell lines, that viruses that we have
only recognized in the last decade are certainly
found. For instance, well HHV-6 isn’t a transforming
virus, but there are cell lines that carry HHV-6 that
have been used in the lab for many years. The same is
true for HHV-8, which is a transforming virus.
So there is a concern that we may have
cell substrates that are contaminated by other tumor
viruses.
Tom, would you like to pick up?
DR. BROKER: I think we have actually a
wonderful opportunity for a so-called natural
experiment. That is solid organ transplantation. It
turns out, as we all know now, virtually all kinds of
organs, not only the corneal we have just heard about,
but kidney, liver, pancreas, part of the intestine,
heart, lung, so forth, have all been transplanted. I
think the opportunity is that the recipient is
invariably immuno-suppressed until the transplant
takes, and then they are slowly weaned off the drug like cyclosporin.
Yet on other occasions, the transplant fails for one or another reason. One could go back into failed transplants to look for the reactivation of agents that came from all these different tumor or tissue types I mean.
One example I could cite that we recently encountered in the course of our kidney transplant study is a pair of kidneys that went in from a five-year-old boy to a 19-year old female. Within a few days, the kidneys had completely become destroyed, necrotic.
It turned out—they suspected CMV infection, but it turned out to be adenovirus. The presumption, and I’m being completely hypothetical, is the five-year-old boy who had died in a bicycle accident, the donor, probably was in the age bracket
where adeno was just a natural infection in his airway, and that these cells say from his tonsils or adenoids, which were in the midst of processing the adeno, became circulating, were in the kidneys, and the recipient female then acquired adeno-infected kidneys, and upon transplant to her, the virus reactivated and just wiped out the tissues. I might also say the different individual who received the boy’s liver also lost the liver. So presumably these were entering through B cells that were in any of these remote organs. Nonetheless, the basic opportunity to look at organ recipients I think is the experiment to ask how much infectious agent is being transferred.

DR. RUSSO: Carlo Russo from Merck. I think as you indicated, these patients are profoundly immune-suppressed. Therefore, is going to be very difficult to assess where this agent came from. In your case, it’s very well possible that the woman was exposed to adeno virus. Since she was immuno- suppressed, that’s the reason why she got the
infection.

DR. BROKER: Well, in that case, it’s why I did also point out that the liver who went into a completely different boy also wiped out. But I agree with you. One doesn’t know whether it’s endogenous, but it does give some indication of infectious agents in these organs.

DR. ONIONS: Could I just actually take a backup actually? I was about to go back to the primary cell issue again. Phil gave I thought a wonderful presentation. I hadn’t heard parts of this before. It actually started to worry me a little bit actually.

To what extent do you need now to control the kinds of colonies of these particular primary colonies? I’m not sure, I mean I don’t know what kind of testing goes on in these colonies for a range of adventitious agents.
Can you maybe just comment on that? I mean are we dealing with inverted SPF animals?

DR. MINOR: Well with respect to the primate, you will certainly not. But they are increasingly heavily monitored. It depends very much on the manufacturer and how much monitoring they do. One manufacturer, for example, has only recently, well in the last four or five years I guess, started using colonies of monkeys that were monitored for foamy virus. The result of that is being revolutionary in terms of the number of cultures that you get surviving to production.

You would have thought you might have started this a bit earlier perhaps. But you couldn’t call them SPF, but they are increasingly closely monitored I think. Certainly some manufacturers have them more closely monitored than others.

But one of the difficulties with the whole of adventitious agent business of course is you only
really find what you are looking for. That’s an ongoing problem.

Things like chickens are a different matter. I mean I think this would establish what you need to do to make an SPF chicken colony. But primates are much more tricky.

DR. ONIONS: There are other cell substrates out there that are used, cells like primary hamster kidney cells in JV vaccines and various other things. So there are I think other vaccines out there that are going to come to attention because they use primary cells. I think we perhaps ought to start thinking of the kinds of procedures that are needed, like closed colonies and embryo derivation of these animals in some cases.

Could I move to perhaps the third element.

That is, we have heard a lot about retrovirus. Retroviruses always come back to focus when we deal with cell substrates. What is the panel and the
group’s feeling here in general about the concerns of using either immortalized cells or transformed cells, because frequently those—well, that’s not an accurate statement. Activation of transcription of endogenous genes is more frequent in such cells. Is that of concern or not a concern? Or do we have to go cell by cell, species by species, to answer that question?

John, would you like to make a comment?

AUDIENCE MEMBER: Well, I think clearly there are some famous cases of activation of transcription of endogenous retroviral genes and genomes and tumor cells. We heard about germal tumors. We have heard about recently in the news about mammary tumors and probably a variety of others.

It is not entirely clear to me whether this actually represents activation of transcription of these cell lines or a fixation of a differentiated state which itself is what’s activating the transcription. I am inclined to think probably both
are true in different cases.

One of the things that we have learned, at least from human tumor cell lines, is that none of these things that are activated have ever been shown to be infectious, despite the fact that as came out in the earlier talk, the probability for recombination between a large variety, for example of the HERV-K sequences, would seem to be rather high. If these were other types of retroviruses, such as MLVs where you do get that endogenous, you do get that kind of thing. So there’s probably something else that’s protecting the people in the cell against actual infectivity in this particular case. We don’t yet know what it is.

DR. ONIONS: I was just going to bring in, I was very struck by the HERV-K story. That is to a very pragmatic level. I take the point that it does seem to me that it doesn’t much recombination or much
adjustment to me, at least into a more functional virus. I mean should we simply be screening any human cell substrate for the expression HERV-K? Would that be something that would be useful to do?

DR. PALLEY: That’s a problem, in that you will I guess you will have a hard time finding a human cell line that would not express any human retrovirus, so I report that it’s the case for HERV-K, the special HERV-K family.

DR. ONIONS: But that particular locus, I mean that is not expressed, as I understood you, in all?

DR. PALLEY: We do not know whether that particular locus is expressed in some cell lines. We know that if this, at least this HERV-K family, is activated for some reason, that there is very likely not only one locus activated within the genome, but that there are several loci that will be activated. So it’s possible that this locus is also activated,
but we also then have the problem that if that locus would not be activated, that there’s some transcomplementation. So one gag, intact gag gene would complement another intact pol gene and so on.

So that could be a problem, but we see it then in several, even in normal peripheral blood lymphocytes, we have HERV-K expression. It obviously is not a problem.

So I wouldn’t see so much a problem in that we have HERV expression if defective sequences are expressed, and that are not coding the intact.

AUDIENCE MEMBER: I think we have to focus on infectivity here, because if we just go looking for expression of defective stuff, you always find it, and we all go home and won’t be able to do anything. It might not be a bad idea perhaps, but --

(Laughter.)

AUDIENCE MEMBER: Sounds good to me. But just to push this point a little bit further on the
HERV-K, you know, about two-and-a-half years ago, there was a report in Cell, which is a better journal than I usually publish in, which claimed that HERV-K env can act as a super antigen that then stimulates diabetes mellitus in some people.

DR. ONIONS: I think that story—just before you go on, I could be wrong, but Johannes might know. Is that story being modified? I’m not quite sure that’s—yes. It’s no longer supported.

AUDIENCE MEMBER: Okay. I think it would be worth explaining how it’s been modified.

DR. ONIONS: I think the retraction is in fourth hit. So before you sort of put that out as a paradigm.

DR. LOEWER: I think the main point is that a couple of groups tried to repeat this data, and they were not able to repeat. So it seems not to be specific and even not effects on the T cell lines could be repeated. So let us depart from this idea.

DR. ONIONS: Can I maybe get your opinion?
I really would like to get some feeling because we have heard a lot, and it’s scientifically really interesting by the expression of these human endogenous retroviruses. I think John has probably just summarized it. It looks like we’re saying the list, this collected group here of retro, are saying that as far as we are aware, at the moment these are not of concern and uninfected, and probably therefore there is not a great deal of point in looking for expression of these in cell substrates, the very pragmatic practical point. Would that be your opinion too, Johannes?

Johannes is nodding. That’s a “yes,” I assume.

AUDIENCE MEMBER: From a research standpoint, it’s absolutely worth pursuing to see if one can find these things eventually. But in terms of vaccine issues, I don’t see how we could possibly deal with it now.
DR. SCHUEPBACH: Yes. I also would like to make a comment regarding that super antigen activity because we are coauthors in that paper. It is true that the presence of these sequences, RNA sequences in the serum we can not repeat, so it’s not specific for IDDM patients. But to my knowledge, our data regarding the super antigen activity and the stimulation of VP cells has not been disputed by any other group. So that is still around. I think that the real important topic here is whether these endogenous viruses actually give rise to infectious particles. I believe that with the PERT assay, we actually can contribute very much to this question. I think, as I pointed out, of course the easiest thing is to test the super natants for reversed inscriptase activity. But I think with a little bit of additional work, it should also be possible to define that profile of RT activity and cellular, DNA polymerase activities along the
different fractions of the sucrose gradient and tend
to recognize any abnormal pattern that might be
associated with infectious rate of viruses.

AUDIENCE MEMBER: Let me just go back then
because obviously the question comes up if the public
is asked to accept a vaccine that’s made in cells that
express HERV-K. Even if one part of the story has
been refuted, the question comes up. You know, are
there potential immunological consequences of the
expression of antigens from these kinds of cells which
are not expressed in human diploid cells? Even if
this story is wrong, I guess, and it sounds like it
hasn’t all been refuted, the question then comes up of
what level of scientific data in the literature is
necessary to completely refute it?
I can imagine the outcry that could occur
if people believe the story. If there appears to be
some controversy about what part of this story has
been refuted, then I think one might have a public
confidence problem as well.

DR. HENEINE: David, I have a comment which could be redundant, but go ahead and say it. While thinking about all these questions from my simple mind, it looks like if you want to compare cell lines versus primary or diploid cells, the two questions that were raised is which ones transmit less adventitious agents or transmit less neoplasms to vaccine recipients. What we have heard so far about the mechanisms of the neoplasms, many of those are mediated by viruses or viral-like elements. So it looks to me that the majority of the concerns are rising from the adventitious agent group rather than from other elements.

So therefore, in trying to make up our mind, based on the available data, which one is the more suitable substrate, maybe we can go very simply with a checkpoint list on these different cells,
targets, which one we can test for the presence of
these adventitious agents known, unknown, and which
can be better monitored, which can be for practical
reasons of culture as well, and make up our mind,
rather than jumping right and left with different
issues.

If you can say cell lines, primary and
diploid, and then go one by one, all these concerns
that we have been talking about, and say which one is
more suitable for each of these points so that we can
conclude at the end. I mean it’s one suggestion.

DR. SCHUEPBACH: I would like to come back
to the human endogenous viruses. Since we all carry
them and have them expressed in one or the other part
of our body, I do not think that this presents a
particular risk. Independent of whether you have a
super antigen activity or not in some of them, I think
is just the same as receiving blood from any person,
because they also would have these endogenous
retroviruses.
So I think the only thing that matters is endogenous retroviruses from other species, not from humans.

DR. LEWIS: This is a question. I think we are sort of being faced with something of a dilemma here, because as you very correctly pointed out, the one way to look for a retro virus, adventitious retroviruses or endogenous retro virus, whatever, is by the PERT assay. Now if we take a situation in which our HERV-K is expressed and there’s RT activity in there with the PERT assay, from a regulatory perspective, what do you do about that?

I don’t think you can dismiss it. I don’t think at this point in time that you could dismiss the use of the PERT assay for looking for adventitious retroviruses or any type of retrovirus activity in there.

So the question is going to be, when you find something, what do you do about it if the assay
is positive?

DR. SCHUEPBACH: I think once you have activity, then you have to characterize what it is.

Depending on whether this is exogenous or endogenous virus, steps will be taken. I think identification is important.

DR. LOEWER: I think we shouldn’t continue to discuss use of this type of cell lines in absolute terms, because the other side of the coin of course is a product which could be made from it. This is always to be judged in conjunction, in my opinion. For example, there’s the question of endogenous sequences which may be active, just retro virus or line elements. So far as I know, this is not the same in all cell lines. It’s mainly expressed in tumor cell lines, and so far as I know, there is so far no need to use these cell lines for vaccine production.

If somebody believes it’s necessary, must
be a very special virus which can only replicate in
these cells, then perhaps we do not have another
choice to use them.

DR. ONIONS: Could I just go to Arifa?

She has been waiting very patiently. Then Steve.

DR. KHAN: Yes, thank you. I think it is
important to clarify the word “expression” in terms of
human cell lines and human cells. I think we all
expect that there will be some RNA expression in the
human cells from endogenous retroviral sequences.
However, I don’t believe that you are going to get
particle production in the majority of the cells under
normal conditions. So therefore, I think the use of
the PERT assay would be very helpful to evaluate
particle production from human cells, which can then
further be investigated for infectivity, as opposed to
looking at RNA expression, which I think you would
find at some level in all human cells.

DR. ONIONS: I would like to endorse that.
I think really we’re making it too complicated. I think it’s very simple. You use that kind of assay system which has a very high sensitivity developed by Yumascript here in the audience. Then if you get a positive, then you go and look and see if there’s something there that’s infectious. It is a hierarchy of testing strategies, it seems to me.

DR. HUGHES: I am willing to take an even stronger line here, which is I think akin to the line that was espoused by John Coffin.

If your technology is sensitive enough, all cells from all vertebrates are going to have endogenous viruses in them, either intact or defective. The question really devolves down to not whether they are there, because they are. If we use the most stringent criterion, are they there, you are not going to be able to do anything. We’ll be paralyzed in terms of making vaccines.

So the obvious criteria is not whether or not these agents are there, and in particular, I think
23 not whether they would make physical particles,
24 because my strong prejudice is if you look hard
25 enough, you are almost certain to be able to find it.

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1 There are almost certainly ways of looking even more
2 stringently than we look now. In very large batches
3 of material prepared for vaccines, if you look hard
4 enough, you are going to find something.
5 The question is whether there is an
6 infectious agent which represents any kind of
7 pathologic threat when its present in a vaccine. We
8 have, as has been pointed out earlier, given from
9 chicken cells, some of which were clearly contaminated
10 with agents which are infectious for the chickens, if
11 not for humans, that hundreds of millions of doses
12 have been given, with as far as anyone can tell, no
13 untoward effects.
14 DR. ONIONS: Okay. I would like to bring
15 Jim in, because I am not sure that statement is --
16 well, it could theoretically be true. Maybe if you
get a more sensitive technique, maybe you are correct.

But there is a very strong distinction if

you look at cell lines like MRC-5 and compare them to

what you see in say AB, you get signals, if you’re

looking at MRC-5 you don’t. If you look in tetra

anacells, you get a signal.

DR. HUGHES: I would argue that is with a

particular assay that has been tuned up to detect RT

in a particular way. I would be willing to wager that

if we look hard enough, we could certainly find

evidence of particle production in any of these cells,

simply because they are full of endogenous viruses

that—I mean the very fact that there’s obviously

eexpression is RNA present.

DR. SCHUEPBACH: But the important thing

is I think the number of particles. You have very,

very low number of particles, and you do a test, a

PERT assay from a concentrated material where you

pellet virus, let’s say, from one liter, and assay
that and find a very low activity. You know for
production of the viral vaccine, the cell harvest, the
vaccine harvest will be diluted 50 to 100. You also
realize that most of the particles even of infectious
retroviruses are non-infectious. Then you certainly
can come to some calculation which permits you to
establish a level of safety where you have a very high
probability that this vaccine is safe.

DR. HUGHES: But the safety is not
predicated on whether or not there are physical
particles. The safety is predicated on whether or not
the particles are infectious.

If the particles are infectious, a very
small number is very important. If the particles are
not infectious, in particular, if the particles are
not infectious for humans, the presence of a
relatively large number of particles is probably also
irrelevant.

DR. ONIONS: My only caution about that is
that you can make assumptions about affectivity that
also are not true. Since Clive Patience is here --

DR. HUGHES: I think you have to do the
test. I don’t think you can make assumptions about
it.

DR. ONIONS: Well, the point I was going
to make actually was that Clive’s group and our group
showed that you could actually infect cells with PERV,

yet those experiments have been done 20 years ago, and
been done by very good people, including George Tadai,
and were unable to show infectivity. It’s just the
techniques have changed slightly and we could get
infectivity.

So I think that you are making—there is
a straight yes or no about infectivity. That is not
always the case with these retroviruses.

DR. HUGHES: I am not trying to suggest
that the assays that we have for infectivity are
necessarily always 100 percent accurate. But what I
want to get away from is the idea that the presence of
a physical particle is somehow a measure of safety or lack of safety.

I certainly agree with you that there are numerous technical problems in determining infectivity. But what I believe we should focus on is better ways of doing infectivity assays rather than better ways of doing physical assays. The physical assays can be very useful if they are coupled to infectivity. In fact, I believe that was a statement that John Coffin made. What I would propose is what I think John Coffin proposed, that we actually use these really wonderful sensitive techniques, but in the context of measuring whether or not the viruses are infectious, not whether the particles are physically present.

DR. SHEETS: Can I ask a very pragmatic question of Dr. Hughes?

DR. HUGHES: Sure.

DR. SHEETS: I’m Becky Sheets, FDA. What
I hear you suggesting is that rather than testing for RT activity by a physical assay, as you called it, a PERT assay or a conventional RT assay, you think that it would be preferable to test vaccines for infectivity assays?

DR. HUGHES: The ability to transmit that RT assay to a reasonable recipient cell. I believe this is exactly what John --

DR. SHEETS: The pragmatic question is that would you do this testing lot by lot on vaccines? For instance, if you were making a vaccine in a primary cell substrate, for instance an egg, would you test each lot of vaccine or each batch of vaccine for infectivity assays? Then the really pragmatic part of it is, if you are making a flu vaccine, where the timing of production, the timing of testing, and the timing of lot release is very tight, would you recommend these infectivity assays on lot by lot for primary source?
DR. ONIONS: We’re running out of time.

Do you want to answer that? You have been asked a question, do you test lot by lot?

DR. HUGHES: Very simply, if we’re talking avian systems, I think there are reasonable ways of determining that endogenous avian viruses are not infectious. My personal bias, and I mean it no more broadly than that, is in the case of avian viruses, as long as you carefully establish that the avian viruses that are present are not infectious for human, that’s not necessary. But that is my prejudice.

DR. SHEETS: That’s fine for SPF situations. I am asking this question because this is what sponsors ask FDA. So they want to know do we need to do this lot by lot, or if a cell bank, you can do a one-time characterization. Of a primary system, you can’t do it that way.

DR. HUGHES: Use SPF chickens and don’t ask.
(Laughter.)

DR. LOEWER: I would like to make a comment to Dr. Hughes’ comments. They are very sound in a scientific meaning, but they face regulatory problems, the main problem indeed. Regulatory authorities have to show that there is no infectivity and the proof of non-activity is always nearly impossible in a scientific sense. You will always find reasons to say you were not able to find infectivity. Look at HIV. If you would use MRC-5, for example, or a lot of other animal cells, you would never find infectivity of HIV. The same is true for many situations.

So there a fundamental problem is to test for non-activity or noninfectivity.

DR. ONIONS: I would like to stop this because we are running out of time and there are other issues.

I am going to take the Chairman’s
privilege and just say that I actually think you need multiple techniques, because I think as Johannes has just said, if you have complete infectivity, you will miss things as we would have missed cell lines producing ver. I think you really need to have a combination technique. So I think it’s a belt and braces situation. That’s a personal view.

What I would like to move onto, is were asked by the FDA also to consider species of origin. I think really you end up in very general statements here. You can argue that if you are worried about adventitious agents, then clearly there are species barriers to the transmission of some agents. On the other hand, other agents do go across species barriers, sometimes in abortive replication. They can be very nasty. We of course know that herpes B going across species barriers is actually lethal. Ad 12 in hamsters is oncogenic. Equine herpes veras, which is an alpha herpes veras is oncogenic in hamsters. There are natural examples of cross-species transmission,
the ovine herpesveras 2 is innocuous in sheep, but it
kills cattle. So there are examples of these
heterologic transmissions being worse than natural
infections.

Is there anything that we can say, the FDA have asked us, in a general statement about species of
origin? My own view is I don’t think you can, but
does anyone want to make a statement?

DR. MINOR: I think sometimes it is better
and sometimes it’s worse.

(Laughter.)

DR. ONIONS: Yes. That’s exactly what I think. Thank you, Phil.

I would like to drop the discussion now,
because I think that sums it up. I’ll turn the phrase
back on the edge and say it’s a case-by-case, it seems
to me.

I don’t want to trespass really on
yesterday’s, but I think maybe just to come back to --
we’re going to move onto assay systems in a second,

but I think one of the issues we’re coming around to
in a second is latent viruses, because those seem to
be the real concern. It may be worth just remembering
some of the things that were partly discussed over the
last two days. That is, that the complementation of
defective viruses can occur. For instance,

adenoarectus can be complemented by HPV in hela cells.

We talked about psuedotype formation both
today and yesterday. I think just I would like to
make the point about pseudo formation. We talked a

lot about retrovirus retrovirus pseudotype formation,

but this can occur across viral species. For
instance, paramyxovirus is rather badly, but they can,

type retro viruses. So you could alter the
host range when endogenous agent is expressed in your

cells.

Of course there are recombinants. Some of

these recombinants, and we have got representatives
who did the work here, interesting recombinants like SV-40 adenovos recombinants.

One of my concerns, we’ll come on in a second, I think is like polyoma viruses in cell substrates and that potential for interaction with other cells.

So if we can take that as a kind of background, can we turn to a question of—this had come up and was discussed by several candidates, unknown viruses. What are the potential candidates and what kind of systems do we use to try and go looking for those unknown viruses.

Anyone want to comment on what we should be doing about novel cell substrates and you have got a virus there that you don’t know anything about.

What sort of technique should we be applying?

DR. PALLEY: I would like to make a comment from that HERV field. I think if we talk
about possible recombinations of HERVs with some other
viruses that might be a little difficult, I think it
could be conceivable.

We do not know, however, it’s very hard to
predict the outcome of whether it is possible at all.
We basically have or we might have situations where a
HERV is expressed where another virus, retrovirus is
also present in the same cell. I think that has to be
discussed or taken into consideration, that there
might be possible recombinations between HERVs and
some retrovirus status put into a particular cell.

However, I think it’s very hard to predict whether
there’s any possible recombination and what the
outcome of that recombination might be.

But I think there are examples where retro
viruses indeed recombine with each other and produce
some productive outcome. But it’s very hard to
predict what, in which ways HERV case for instance, or
whatever HERV sequence within human genome could
recombine with something else.

DR. ONIONS: I was just going to point out
I think one of the things I have been very struck by by some of the talks here, particularly the polymerized talks, it does strike me, the comment I made to Phil earlier about the use of cynomologous monkeys and perhaps have these been screened for other polyoma virus. That seems to be of concern. We know that polyoma viruses are coming in as contaminants in bovine serum into the primate cells, the bovine polyoma virus. They worry me as potential adventitious agents. I am just wondering again whether we should be doing more in terms of redundant PCR approaches to look for these agents in both primate and non-primate cell lines.

Any views on that?

DR. ROBERTSON: This is potentially the most important door, also the most difficult to deal with. If you think back to what Phil was saying in the first talk this morning, all these instances of
vital contamination, generally they occur with viruses
unknown at the time, viruses expected, the presence of
viruses in vaccines or biological preparations.
Potentially is not something we’re talking
about today, it’s not an endogenous virus or
recombination between an endogenous virus, but
something unknown that’s going to leap up at us out of
the dark. Of course almost impossible to deal with.
But Joerg was saying this morning about
this is what we should be looking out for, the

unknown, and if possible using a more broadly reactive
type of assay rather than highly specific type of
assay to look for something.
If we knew to look for something, that’s
fine, we can deal with it. It’s what’s not there
which causes the problem.

DR. SCHUEPBACH: May I add something to
this? I think our chances to detect such unknown
agents are really much better if they are present at
high concentration. So I don’t know whether you accept that concept of cellular cloning in order to either get rid of these agents or to have them at the very high concentrations so that their detection is actually much easier. In the meantime, you can try to activate the host cells by all kinds of different agents. You do EM studies, you do serological studies, use broadly cross-reactive antibodies. I mean this is a wide field actually of methods you can employ.

So I think using such an approach, we should actually be quite capable of detecting such agents.

DR. MINOR: Can I ask for a definition here? What do you actually mean by a virus? I mean what I would understand by a virus is something that actually grows in the cell and increases in numbers.

If you think back to the very, very early days of polio vaccines, amongst others, there was an awful lot
of effort put into trying to make sure that you put
your supernatant or whatever, into all sorts of
potential different systems where a virus might grow.
So you put it into different cell
substrates and you look for cytopathic effect, where
you can say maybe you have got a virus that doesn’t
cause a cytopathic effect. You put it into mice, you
put it into eggs, you put it into everything. So it’s
like evidence of actual growth. You see?

I think there’s actually quite a lot of
effort that goes into trying to detect viruses that
you don’t know are there, but you suspect might
actually grow in some system that you are going to
check it on. If they don’t grow, I’m not sure certain
that you are worried about them, or even if they are
viruses. So what do you actually mean by an unknown

virus?

DR. ONIONS: Well, okay. Let me give you
an example. I think one of the areas that is going to
emerge as a concern are going to be circaviruses. The
reason I say that is that TGV may be a circavirus. We now know of others. Thomas was talking about the kind of normal flora of HPVs. It looks like we all have a normal flora of these circaviruses. Certainly when we started now looking in animals, we find these all over the place.

So I think they may come up as potential cell substrate contaminants, but also in our serum, in our trypsin, and so on. Of course trypsin is full of of course circoviruses.

What we know is if you look in a cell substrate like PK-15, which has used in the pig vaccine industry, that carries a circovirus genome. It looks, it’s possible semi-defective, but you can introduce it as an infectious agent, but most of the time it isn’t inducible as an infectious agent. So I think I’m not quite sure I take your distinction. I think there are latent viruses there that are reactivatable under certain circumstances and
are a concern. In fact, in the vetmurines vaccine, you have to get rid of that virus.

DR. PALLEY: Yes. I just had sort of an alternative. Virtually all the conversation has revolved around using higher eukaryotic type cells as the producer cell source. There is an alternative that’s being actively pursued in the papilloma vaccine business, which is to make virus-like particles either

with a baculo virus expression system in insect cells or even in E. coli.

Very briefly, what’s done is the L-1 and L-2 capsid proteins of papilloma virus self assemble no matter where they are over produced. Four different groups are pursuing these now. I am sure they are engaged in some corporate relations. But one of the neat strategies that John Shiller here at NIH and others have done, is to fuse peptide epitopes or even intact other genes to the L-2 protein at their n-terminus. It turns out that when
L-2 assembles into the virus-like particle, but brings this n-terminal protein in with it, that other protein is on the inside of the virus-like particle. So it will ultimately be presented to the recipient of this virus infection. It’s non-genetic, but it will be taken up by cells. So there is in effect no risk of some bacteria phage infection running rampant in our bodies. So it’s a complete alternative.

DR. ONIONS: I think obviously there is a move to sub-unit vaccines in this form of vaccines. But I think it’s still going to be a long time before our traditional eukaryotic cell production is going to be lost. But I take your point. That is a valid point.

Could I just ask perhaps questions about we had examples indeed from Thomas toady about using redundant PCR techniques. It would be feasible to screen for a number of viruses that are of concern, like herpes viruses, circoviruses, polyoma viruses,
herpes viruses retro viruses by redundant PCR techniques. These are not in FDA terms, validated techniques. On the other hand, they are very powerful techniques. For instance, one of my colleagues just developed a herpes virus redundant PCR technique with 112 primer combinations that to date has picked up all of the herpes viruses that’s been challenged with both human and animal origin. So you could go looking for herpes viruses by that kind of technique. Robin Weiss’ group in London picked up a new human herpes virus, HRV-5 by a redundant PCR technique. So do any of you think that these kinds of technologies should be implied to cell substrates? If so, which viruses should we use? Because it is a lot of work to do this kind of thing. So should we be doing this or is it not necessary? Can I have comments?

Jim, you look like you are about to say something, but not quite sure.

DR. ROBERTSON: One would not expect to
see this type of assay risen up in a pharmacopeial recommendation of any kind. But certainly they do have uses at the investigational level, especially with some incredibly novel cell types. Everything is kind of going molecular these days. All these assays are looking at things from the very molecular point of view, and picking out a signal say with your herpes primers, need not necessarily say that you have got an infectious herpes virus. So go back to this argument we have been having this morning about it’s infectivity which potentially is what we’re concerned about, and not picking up a fragment of a genome. These are assays, again I mentioned that Joerg mentioned them this morning, broadly reactive molecular assays. Phil rightly pointed out that we have had in place for eons broadly reactive infectivity assays using tissue cultures, suckling
mice eggs, looking for signs of infectivity of any kind.

DR. ONIONS: But don’t you think—my feeling about that is, I tend to share, I don’t think one would ever use these kind of assays on a routine basis.

DR. ROBERTSON: No.

DR. ONIONS: They might be useful in establishment of a master cell bank or something, you know, the first one. But I mean I would criticize, I don’t think that current infective assays do pick up everything. I think that’s the whole problem. I think, for instance, that it would miss—well, polyem virus has perhaps been used.

DR. ROBERTSON: At the end of the day, that’s the weakness of anything, that you will not pick up something that’s not designed to pick up. You will potentially miss viruses in an infectivity assay.

But you also potentially miss viruses with a redundant
PCR. If you don’t pick something up, you can’t say it’s there or not there. It’s a bit of a philosophical argument.

DR. ONIONS: That’s why I’m just saying shouldn’t you have an adjunct to these? I mean I think should you not have at least an adjunct in terms of broadening the kinds of assay systems that you are using?

DR. ROBERTSON: Sure. Oh yes.

DR. ONIONS: Does it have anything to do with the—I mean there are people out there who have to do this for a living, rather than us who can just sit here and pontificate about it. How does this go down with the industry? What does the industry feel?

DR. PALLEY: Just one point regarding again HERVs.

DR. ONIONS: Sorry?

DR. PALLEY: I mentioned in my talk that HERV-W family that has been reported for the first
time this year and has been isolated from vitro by

particles from multiple sclerosis patients. It turned
out that it at least codes for an env gene. So it’s
certainly worth—the human genome I guess is among
the genomes that regarding endogenous retroviruses is
among the best characterized genomes besides mouse,
for instance.

I think it is certainly worth to continue
and even by such redundant PCR approaches and so on to
further characterize HERV sequences on endogenous
retrovirus sequences, and to see, to give then an
estimate whether there are any additional sequences
that could be harmful. So far, we did not find any
sequences, but it’s certainly worth doing that.

AUDIENCE MEMBER: I think if you just kind
of look back at the history of biological products,
and maybe even going way back to when hepatitis B was
discovered, and then there became the ability to look

for hepatitis B in blood and blood products. There is
always concern about what are you going to find, and
what are the implications, and what are the costs, and
all of those things.

But the bottom line is as technology evolved, then the discovery of reverse transcriptase,
and when I was still here at the agency, we applied in a research setting to vaccines and first demonstrated
RT in yellow fever. You get concerned about again, what are you going to find when you look at all these
cell substrates, and then with the more enhanced sensitivities of these systems. You are always in the
same muddle. That is, is this appropriate to apply across the board? Where should it be properly applied? Should it be done more in the initial stages of characterizing something versus a routine quality control test?

Those things, you can’t sit here in a meeting or on a panel and give specific answers to those questions. I think the bottom line is as technology evolves, and it’s going to continue to
evolve with more sensitivity and specificity

hopefully, it needs to be explored and it will find

its appropriate place in the overall testing, whether

it’s in characterization or perhaps in some cases, if

it’s appropriate, on a routine basis.

But I think the general principle of

applying new technology as it begins to be available
to look at these issues, particularly as they relate
to safety and the presence of adventitious agents, is

unassailable.

DR. ONIONS: I agree. Can we move on,
because I would like to just cover TSEs just before we
have lunch time. I’m desperate for some lunch.

If you have got something, sorry to
inhibit you. If you can be brief.

AUDIENCE MEMBER: I agree completely with

what John says, but would add that if we are faced
with a decision of whether to approve the use of
different types of cell substrates that are
tumorigenic or derived from tumors from which we don’t
know the mechanism of transformation, we are faced
with not only the question of should technology be
applied, but is the technology as it exists today and
can be applied today, good enough to permit us to say
that it’s okay to use these cells. So that is a very
practical question which perhaps could be answered.

DR. ONIONS: Can we have a view on that?
That is, given the technology we have today, is it
acceptable to use the kinds of substrates we have been
talking about, that is tumorigenic or immortalized
cell substrates? Are we confident that with the
technology we have, that we can use these cell
substrates safely?

DR. BROKER: I would just basically say I
 think so. I think if we combine PCR with these
microchip or microprobe arrays, DNA chip technologies,
and we have the growing human genome base and the
9 analogs and a number of other species, I think we have
10 got the tools at a level of sensitivity far beyond
11 what would probably would be more than adequate.
12 DR. ONIONS: Okay. I would just like to
13 finish up, because we heard a really I think important
14 interesting talk from Neil. While perhaps the risk of
15 spongiform encephalopathies in the kind of cell
16 substrates we are concerned with is probably extremely
17 remote, the consequences of being wrong about this
18 issue are potentially devastating. So it is certainly
19 worth cautious consideration.
20 Really I think Neil in his talk, already
21 summed up these key issues about the possible origins,
22 are the mutations spontaneous or infection, and the
23 kinds of cell substrates of concern might be, it seems
24 to me, are the neuronal cells. Since it’s recently
25 shown that in the peripheral introduction of TSEs, the

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1 B-lymphocyte might be important to carriage, then
2 perhaps lymphoid cells, particularly B cells that are
invariably used, and since we’re looking at the possibility of using HIV and T cells, maybe that suddenly becomes an issue. Maybe we should be looking at lymphoid cells for the potential of there being spongiform encephalopathies.

Which brings you back to the question that Neil finished with. That is, what should we do? It did strike me that one of the possibilities was that there are now very good, very interesting new cell lines being based on retinal cells, which we heard from Dr. van der Eb and others, which look very, very promising for the generation of anti-viral vectors.

But as they have, as I understand it in theory, I mean just at the simplistic level, should not one thing be done and just sequence the PrP gene in that? The probability of having a key mutation seems to me extraordinarily remote. But then it’s a relatively simple thing, cheap thing to do is to go and sequence the PrP gene. Is that something that we should do in that kind of a situation? Should we also
do that in T lymphocytes? It’s a trivial thing to do?

DR. CASHMAN: I would say in this case, you get what you pay for. It is a trivial experiment,

but if one out of a million or one out of a thousand cells could be harboring a mutant prion protein gene, the technology is a little more dicey. So yes.

DR. ONIONS: I was thinking of excluding the origin of the familial form, which clearly occurs to some inherited disorders. I mean I think you are right. You can’t cope with a somatic mutation.

DR. CASHMAN: Okay. You can’t cope with somatic mutation, I agree. But certainly one can cope with a mutation that’s in every cell, yes.

DR. ONIONS: Neil touched on validation technology. I think that’s important. I think there are new techniques for doing validation of TSE removal, but using a disrupted PrP protein. But I don’t think that’s going to be applicable to quite a lot of the processes that are used to produce vaccines
at the moment. It is by technology products, but not
to vaccines.

I just wanted to touch on testing because

I know that Neil has an interest in that area. It
seems to me that we were sort of rather optimistic a
couple of years ago, and indeed, there have been
publications by Bruno Esch and others on specific
antisera of PrP Sc. But those haven’t held up. They
actually pick up aggregated protein and not, as I
understand it, strictly PrP Sc.

We can use, and we have been using,
treatment of protease followed by immuno blotting. It
certainly works, but it isn’t that sensitive. The
problem, it seems to me, is that we really don’t have
a specific test that’s an in vitro test. The only
thing that you are left with, at the moment, it may
change, but at the moment is animal inoculation.

Would you like to comment?

DR. CASHMAN: I think that the bio assay,
with no species barrier, can detect one unit of infectivity. It's the most sensitive thing we have to date. But in fact, there is new technology. Mary Jo Schmirr and her colleagues have developed a very sensitive capillary electrophoresis technique which is, if one can believe the papers, including one in press, is as sensitive as bio assay. So technology is evolving, and there may in fact be a specific and sensitive test for PrP Sc right around the corner.

DR. ONIONS: Okay. On that optimistic note, I am going to wrap the session up. I think we're all getting edgy for lunch. Unless anyone has got some burning issue that they wish to go into before we go. If not, I think the panel members, and thank you all for participating.

CHAIRPERSON RABINOVICH: Thank you, Dr. Onions. There is a light repast outside for those that have been so patient. I would like to get
everybody back in here in 15 minutes.

For those of you who would like to avoid the wholesale garage sale that’s going to go with your luggage, for those of you that haven’t checked out of your room, I encourage you to complete that now. Thank you.

(Whereupon, the foregoing matter went off the record at 12:33 p.m. and went back on the record at 12:53 p.m.)

CHAIRPERSON MYERS: Back to order. I would like to introduce a co-chair and a new person we are very pleased to have attending the meeting, Dr. Gary Nabel, who is the new Director of the Vaccine Research Center at NIH. So he is going to join me in chairing this session. I am probably going to disappear before the end of the session to make a plane.

The first night I got here, as some will recall, I came in a little late. I ran into a couple of you in getting a beer because it was after the time
of the close of the meeting. The discussion ensued as to what is a designer cell substrate. What do we mean by that? My first reaction to that of course was it’s anything that I happened to have made. Clearly at this point in the meeting, it’s not primary cells. I suppose from a strictly semantic perspective, it would be a cell substrate created with specific characteristics. It could be immortal or not. But I think over the last couple of days, at least my thinking on this and for the purposes of this discussion, by a designer cell substrate, we mean a cell substrate of defined origin and with a defined pedigree. It is probably immortalized because it is likely to have been cloned. It will be validated as specific pathogen-free and at least specific pathogen sought and perhaps in certain circumstances, defined as non-infectious. For the purposes of the next discussion,
we are really talking about immortalization. Jim

McDougall, as you know, presented his paper yesterday.
So we’ll start this session with the first paper by Dr. John Sedivy from Brown University, who will talk about differences in the capacity to immortalize rodent, primate, and human cells by tissue culture passage or viral transformation.

DR. SEDIVY: Thanks very much for the invitation. I am sorry that Jim gave his talk yesterday because—well, maybe it will jive all together.
I was asked to give somewhat of a historical overview on the issues of replicated cellular senescence, and obviously the topic of cellular immortalization.

So from a historical point of view then, this is the Hayflick phenomenon. This experiment has been performed in numerous labs and always with the same result. This happens to be an experiment in my
lab. You will see a number of slides like this from me today. What we’re plotting here is replicated lifespan, the doublings of the culture versus days.

We see a culture growing and then reaching a non-proliferative plateau. This is what we define as senescence.

Really the interesting point here is that the correlation here of this plateau is with the number of cell divisions as opposed to chronological time. The question that has been plaguing this field ever since its inception is well, is this really some type of a terrible artifact. I don’t really want to get into this discussion. It really revolves around

the issue of media and media artifacts, and have these really been adequately resolved today. I don’t think they have, especially for some more specialized cell types. I think they have been pretty well resolved for keratinocytes, maybe breast epidermal cells,

fibroblasts, et cetera.
One really has to keep in mind that if one sees a culture that is slowly declining in its proliferation, this could simply mean that increasingly a larger and larger fraction of those cells are withdrawing from the cell cycle. This could be perfectly explained by inadequate culture conditions, such that eventually on the macroscopic scale, the culture has ceased proliferating.

There are really three arguments that have been used historically to justify the claim that replicated senescence is a biologically interesting phenomena. Here we are plotting, again very simply, the mean-like span of a species versus fibroblasts replicated life span in tissue culture. As you can see, there is a rather striking correlation, such that animals that don’t live for very long don’t have cells that live for very long in tissue culture.

The next phenomenon that one often sees cited is the age of the donor plotted against—here
is the age of the donor, and the remaining life span of the cells, in this case fibroblasts taken from that donor. As you can see, the points are all over the place. In fact, more recently, this view has been challenged by a recent paper in PNAS from Vince Cristofalos, who actually claims that this correlation doesn’t exist. But if you read the literature, you will see this coming up over and over again.

The one fact that seems to remain, at least to my knowledge, and that is if you look at these points down here, these are fibroblasts taken from individuals that suffer from premature aging syndromes. These are called progerias. Typically, these cells have a very short life span.

So this really is the issue here. How do we differentiate between senescence, quiescence, and differentiation. I think that for the purpose of discussion today, this is really not a point of major interest, but for historical reasons I’ll go through it rather quickly.
Quiescence is defined as a reversible process. So what we are talking about here is essentially a cell cycle phenomenon. That is, we can have a culture that is cycling or contains a large fraction of cycling cells. Then these cells can withdraw into the quiescence state. Then when they are induced with the proper growth factors, and here of course the key phrase is what are the proper growth factors to elicit this phenomenon. At any rate, we are talking about a reversible process. Whereas senescence by definition is irreversible.

So then of course the very interesting next question is how do we differentiate senescence from terminal differentiation. I don’t really have answers here because in many cases, this is very difficult to do in many specialized cell types. What one would like to see in general is the absence of features that are characteristic for terminally differentiated cells. But this is not possible in
many cases.

So really this has given the impetus to a search for molecular events. So then if we pose the question are there molecular events that are unique to senescence versus quiescence versus differentiation, again, the picture is not very clear cut. I don’t want you to absorb this whole slide. Suffice it to say that this is well, not all, but the major part of the regulatory circuitry in G-1. Here you see the D-type cyclance. CDK-4 and CDK-6 driving RB phosphorylation, which in turn drives the second phase, which is cyclin E production, activation of CDK-2. Of course there are a lot of modifying proteins here, CDK inhibitors, kineses that activate the basal CDK kinase, et cetera.

Now this is an area that is receiving a lot of attention. The general theme, at least to me, it seems that there’s a high degree of overlap between mechanisms that regulate quiescence, senescence, and
differentiation. I don’t think this is really surprising because all these three states are characterized by the absence of cell cycle progression. In most cases, by an arrest in the G-1 or a G-0 state.

The one central theme is that the regulation of cyclin dependent kinase activity is necessary to achieve a physiological cell cycle arrest. In addition to the cyclins, which are the positive affecters, there is a number of CDK kinase inhibitors that have been shown to play a key role.

The two major inhibitory pathways that act on this basal cell cycle machinery are the RB pathway, shown here, and also the pathway regulated by the tumor-suppressor protein P53. In both of these pathways, CDK kinase inhibitors have been shown to play key roles.

So let me turn to the issue of
immortalization. We all know that senescence can be
overcome because quite obviously, there are many cell
lines out there that are very immortal. So in a very
simplistic and general sense, we can think of cell
culture in three broad categories. We can have
primary cells or cell strains that have a limited life
span and senescence after several passages. We have
a category of cell lines that are immortal, not
necessarily by the 3T3 protocol, but in general, they
display the characteristics of unlimited lifespan,
on-malignant phenotype, and in most cases by the
ability to become quiescent.

Finally, we have the large group of cell
lines that are derived from either tumors or have been
transformed by one process or another. These of
course also have an unlimited lifespan, but they have
a malignant phenotype as defined by one or more
criteria. They also usually cannot become quiescent.

This again is the Hayflick plot. What I
am showing here is a rodent culture, mouse in this
case, and human. This little bump on the curve in
fact is senescence for a mouse fibroblast culture. So it’s been known for a very long time that rodent cells can overcome senescence spontaneously.

You can also see the great difference between the replicated lifespan in vitro of human cells that go on for a very long time. If this experiment here was continued, it would level off and you would see the typical Hayflick phenomenon. So the human plateau up here in fact is corresponding to this rather short plateau senescence in rodent cells. So the relatively low frequency of immortalization—I should point out that this doesn’t really seem like a low frequency, but on a per cell basis, it actually is an event that has a frequency of 10 to the minus 5, to 10 to the minus 6.

It’s just that the X axis is plotted in days here. The fact that this immortalization can be stimulated by mutagens has led to the hypothesis that
this in fact is a mutational event in nature. This is supported by the existence of several viral genes, such as, and we have heard about them here, SV40 large T antigen, polyoma large T antigen, animal virus E1A, HPV E6 and E7, that can cause immortalization. In fact, when these genes are introduced into rodent cells, they are sufficient to cause immortalization in a single step. In other words, if you take a rodent culture and you put SV40 large T into those cells at this point, the curve would look like this. No apparent senescence under the right culture conditions. So what are these viral oncogenes doing to promote immortalization? Without going into a lot of detail, there is a large body of evidence that now indicates that these proteins interfere with the function of the P53 and/or RB growth inhibitory pathways. In agreement, there’s a lot of data from knock-out mice now recently that has shown that the
elimination by gene knock-out of a variety of
negatively acting affecters can result in apparent
one-step immortalization, as shown here for example.

To date, embryo fibroblasts from strains
deleted for P53, P16 inc 4A, P19 arf 1 in P21 cip 1
have displayed this apparent immortalization

phenotype.

So what happens in human cells? Normal
human cells have never been observed to spontaneously
immortalize. Senescent cultures do not give rise to
sub-populations that resume proliferation as shown
here. Treatment with mutagens has been shown to
sporadically give rise to immortalized derivatives,
but the frequency of these events is significantly
lower than that in rodent cells.

Let me now talk a little bit about the

phenomenon of crisis. So what happens when we put,

for example, SV40 large T or E1A into a human

fibroblast? What we get instead of immortalization,
is a phase of so-called extended lifespan. So here we see a primary cell, the initial proliferative phase.

This is senescence or the Hayflick limit. The introduction of a viral oncogene is going to cause an extended lifespan for variable duration, typically in human fibroblasts of 20 to 30 divisions. Then one sees a second proliferative decline. This has been designated as crisis.

Now this decline at the end of this extended lifespan which we call crisis, this word is somewhat ambiguous, because it has also been applied to rodent cells. These cells do not display a two-stage mortality process. So to distinguish more clearly between senescence and crisis, some groups have started to use the word “M1” for mortality stage one, and “M2” for mortality stage two.

Senescence is different from crisis. These are not just the same proliferative decline. The main distinction is that cells in senescence or M1 are truly non-dividing. Whereas in crisis cultures,
the apparent absence of proliferation on the macroscopic scale is actually the result of ongoing cell division combined with ongoing cell death.

This is an experiment that was performed in my lab. What we show here is that elimination of the CDK inhibitor P21 in a pre-senescent normal human fibroblast causes an apparent extension of lifespan that is equivalent in magnitude to that elicited by SV40 large T antigen. So also in human cells now we have been able to do ablative intervention. That is eliminate the activity of certain negatively acting affecters and cause an apparent extension of lifespan. In terms of cell substrate design or the technology that would go into doing this, this was really strictly an aside, we have now developed methods—these are really based on gene knockouts, homologies reculmination gene targeting, that can be used to delete entire genes, multiple genes in human cells, including normal human cells.
So let me now turn to my last topic, which is the molecular clock of aging. I think probably this is where I am going to overlap with what Jim has already said. As I told you, there are some older observations that correlated entry into senescence with the lap cell division as opposed to chronological time. Quite a few years ago, this has led to the proposal for the existence of some sort of a molecular clock. Then one envisioned that the running down of this clock would generate a signal that triggered the senescence program. Then the expression, for example, SV40 large T could either prevent senescence by overriding a signal from this clock or by what I think is more likely now in light of new evidence, actually interfering with the senescence machinery itself. So as you know, the currently prevailing hypothesis is that the nature of the molecular clock is the attrition of telomeres. This is a slide by one
of my dear friends, Chris Counter, who has fancifully imagined H-TERP, which is the catalytic sub-unit of human telomerase sitting here at the end of a chromosome end. So this is a telomere here. Then catalyzing the addition of the telomere heximer. You can see the telomerase RNA that acts as a template for that process right there.

Germ cells and some key stem cells are known to express telomerase catalytic activity while the majority of somatic cells lack this activity. The estimation of telomere shortening for one generation is in human cells between 50 to 100 bay spares. So that’s 50 to 100 bay spares per S phase. This correlates reasonably well with the average telomere length in a young human fibroblast of 18 to 20 kilobases and the length of 8 to 10 kilobases in the senescent fibroblast.

I think it’s an important observation that senescent cells in fact contain appreciable
telomerase. So here we have a normal cell or a young cell. We get attribution of telomerase. At this point, the telomerase are maybe 8 to 10 kilobases in length. This generates a signal. If the cell is now driven into the extended lifespan phase, these telomeres will continue to erode because telomerase is not expressed in that state. Eventually one enters into a crisis which is caused by erosion at the end, genetic instability, et cetera, et cetera.

It is really the nature of this signal that I think is one of the enduring mysteries of the field. One can really now beginning—we can start to see the process as being composed of a clock, a signal, and then the senescence machinery itself which is most likely composed of the same players, CDK cyclin inhibitors, et cetera, et cetera, that are used in other types of responses such as differentiation and quiescence.

The linguistic definition of senescence is the state of being or the process of becoming old.
This term has therefore been used to describe essentially any sort of age-related irreversible proliferative decline. In light of these new molecular insights, I prefer to use senescence in the more restrictive mechanistic sense to designate the response triggered in normal cells. I really believe that senescence is an active genetically programmed process that responds to an inductive signal. Perhaps telomere shortening, but that is not 100 percent clear.

How the signal is generated is not really well understood. One can argue that the ensuing growth arrest has the obvious advantage of preventing the cell from becoming grossly genetically unstable. In contrast then, I think of crisis as an unphysiological state. You have to do something to the cell to drive it to this point, and that it leads eventually to the catastrophic breakdown of chromosome stability, which is caused by critical telomere
shortening on many chromosome ends.

So now this is really just a restatement of the two-stage mortality process. What I have added here now is telomere length in kilobases on the Y axis, the replicative age on the X axis. So here we have a cell in the beginning. If this happens to be a germ cell or a stem cell, it will maintain telomeres because it will express telomerase activity.

Most somatic cells will start down the slippery slope of telomere attrition, eventually entering into a physiological state of growth arrest, through which they can be driven by either the expression of certain viral oncogene or the eblation of certain inhibitory pathways that are intrinsic to those cells.

The cells then enter into extended lifespan. They continue to erode telomeres. They enter into a state of crisis, which is characterized by genomic instability. Finally, at this point, one
can attain a truly immortalized derivative in the key step here, is the expression of telomerase catalytic activity.

I should also point out that telomerase need not be expressed at the final step. It has been shown experimentally that telomerase can be artificially or experimentally activated anywhere along this line, and that that will lead in some cell types, not necessarily all cell types, to immortalization. However, I think the large body of evidence suggests that at least in vivo, and by this I mean during the natural development of malignancy, the activation of telomerase activity is a relatively late step.

So if crisis doesn’t exist in rodent cells, and bypass of senescence is sufficient for immortalization, how does telomerase become expressed in somatic cells, rodent somatic cells? The bottom line here seems to be that telomerase is not very
strictly regulated in rodent cells and tissue. A variety of rodent tissues have been shown to express telomerase activity. Telomerase negative primary cultures often become telomerase positive over time even prior to reaching senescence.

In contrast, telomerase appears to be regulated very stringently in human cells. Therefore, telomerase activation could occur in rodent cells that are undergoing immortalization either prior to or after the senescence bypassing event, and could easily occur in the subtle and gradual fashion so that no clearly apparent downturn in proliferative capacity of the ball culture would be observed.

In other words, one step immortalization that one sees so often in rodent cells may in fact require two steps, the obvious step of senescence bypass and very likely a second step that may be very subtle, at least in rodent cultures. That is, of activating telomerase catalytic activity.
So I think that is about as good a summary as I can think of in 20 minutes. I will be glad to entertain questions.

(Applause.)

AUDIENCE MEMBER: Bill Egan, from the FDA. When you immortalize cells, you know, after they go into crisis or whatever, what becomes the length of the telomere? Does it go back up to 20 kilobases?

What maintains the length of that telomere at a fixed --

DR. SEDIVY: That’s a very good question.

AUDIENCE MEMBER: Why doesn’t it become 30 or 40 kilobases.

DR. SEDIVY: In fact, it seems that excessive telomere length is not good, at least in human cells. It’s been known for a long time that many spontaneously immortalized human cell lines which we love and honor like 293 and Hela, et cetera, et cetera, have very short telomeres. These telomeres can be maintained at a length of one to two KB. These
cells seems to be perfectly happy with that.

So I think it’s more the maintenance of the telomere length rather than the absolute length of the telomere.

If you artificially introduce telomerase catalytic subunits into fibroblasts, what one typically sees is that the best clones are ones that build up telomere length to about 8 to 10, 12 KB and then maintain it at that level. It seems to be a function of the expression level of the H-TERP gene, because if one does this experiment, you see cultures that very slowly erode their telomeres. They will eventually senesce. You see cultures that build up telomeres to maybe 20, 30 kilobase in length. That doesn’t seem to be good for them because the rate of growth goes down. So really the best cultures are the ones that maintain at least in fibroblasts. So I think it’s the maintenance rather than the absolute length.
DR. HUGHES: Would you please comment on Carol Greider’s knockout mice?

DR. SEDIVY: Well, yes. I didn’t get into that at all because that’s at least for the time being -- there are some paradoxes here. Okay? The obvious paradox is that mice have extremely long telomeres, 60 KB on average. This is the laboratory mouse.

Muskulorattus has perfectly normal telomeres, for example.

In fact, this is really the other way around because these cells live for a very short time in tissue culture. When they undergo senescence, they undergo senescence with telomeres that are 50 KB in size as opposed to 60 KB in size.

So there are really two answers to that.

One answer is that if you look at individual telomere ends, you will see that there’s a certain degree of heterogeneity in that in fact in all cells, including
mouse cells, you always see a minority fraction of chromosomes that have very short telomeres. If the signal is caused by a perfectly short telomere that gives, for example, DNA damage-like signal, then you only need one per cell to give the senescence signal. So that’s one explanation. I don’t really know whether it’s correct.

The other explanation is that mouse cells don’t senesce. They neither undergo crisis or they undergo senescence. In fact, that plateau that we are seeing during the immortalization is not senescence. It’s a differentiation-like process. There are people, including myself, that given this kind of loose distinction between quiescence, differentiation, and senescence, would prefer to define senescence now as a process that is triggered by telomere erosion. Obviously when a mouse primary fibroblast culture undergoes senescence, it’s not doing it, probably not doing it because it’s
receiving a telomere signal.

I don’t know if that is—does that make sense?

DR. HUGHES: I had hoped you would comment on the mice themselves.

DR. SEDIVY: The mice themselves? What do you want to know about the mice themselves? They are alive.

DR. HUGHES: Yes, I know. But in the Greider experiment with telomerase knockout.

DR. SEDIVY: If you knock out telomerase in mice, it takes six organismal generations to observe lethality. Okay? What you see at each generation is that the average telomere length. So generation one, it’s 50. Generation two, it’s 40. Generation three, it’s 30.

If you take mouse and real fibroblasts at any one of these generations, they senesce in vitro on schedule. Is that what you wanted?

DR. HUGHES: (Inaudible.)
DR. SEDIVY: Well, I think I just offered you one explanation for that. That is that what we are calling senescence is not senescence. It’s something caused by some insufficiency in the median that is in fact triggering a differentiation event.

Actually, Jim McDougall and I also don’t quite agree about what’s happening in his keratinocyte cultures because what he is calling senescence, some keratinocyte biologists would prefer to call differentiation.

DR. NABEL: Okay. If we could move the questions along, maybe brief answers. Then we’ll move onto the next speaker.

AUDIENCE MEMBER: Alex van der Eb, Leiden. You just already answered, I think, my question, which was why do mice cells, mouse cells enter senescence while they have such long telomeres? In fact, you answered already part of that question.

Do these cells that enter a so-called
senescence have high levels of P21 or P16 or something like that?

DR. SEDIVY: Yes, they do. Yes, they do.

AUDIENCE MEMBER: So there is a signal then.

DR. SEDIVY: If you take a knockout mouse for P21 that doesn’t undergo senescence. It just keeps going. But you know, that’s what I was trying to say. That is that op regulation of P21 is not a molecular market for senescence. P21 is op regular because of oxidated stress, osmotic stress, differentiation signals. This is a very general machinery that is used to establish cell cycleresce. I, in fact, don’t know of any molecular marker that is specific for senescence. This includes the famous senescence-specific betagalactocytis activity. You know, you see a lot of people staining cells, and they turn blue and they say it’s senescence. Everybody knows if you put hydrogen
peroxide on your cells, they turn blue as well.

AUDIENCE MEMBER: Just a brief comment for those people who might be setting up assays that would be monitoring P21 sip. We, as I showed, found that P21 is elevated in a subset of the HPB infected cells.

We did three other related assays. One was to look for P21 MRNA. It turns out it’s abundant in all differentiated cells. But there is a post-translational control on the accumulation of P21.

It turns out what happens is that if there is not a signal that unscheduled in a synthesis is underway, namely, abundant cyclin E, then proteosomes rapidly degrade the P21 that’s translated. When we put in proteosome inhibitors, P21 piled up in all cells and all replication was blocked.

We went on to ask one additional question.

That is, how does P21 actually block S phase or DNA synthesis. Unexpectedly, it had nothing to do with blocking cyclin E activity. It turns out cyclin A,
CDK-2 or cyclin A CDC-2, can phosphorylate DNA preliminary cell and all these other subunits I showed.

The one thing cyclin A can’t do is bind to PCNA. But when the P21 sip piles up in these cells that have excessive cyclin E, the way the P21 is actually functioning is by binding to the PCNA and blocking elongation, not initiation.

DR. LEWIS: This may be a naive question. Is there any change in the activity of endogenous oncogenes in cells at about the time they are entering into senescence, especially rodent cells?

DR. SEDIVY: By activity, you mean level of expression? I am not aware of that.

AUDIENCE MEMBER: What happens in spragues? I mean are they different than muskolorattus? Do they have shorter telomeres that they go through?

DR. SEDIVY: What happens in spragues is exactly the same that happens in muskolor.
AUDIENCE MEMBER: So even though one has

60 KB and one has 2 KB?

DR. SEDIVY: I mean, you know, this kind
of all argues that the length of telomeres has nothing
to do with this plateau in mouse cells that we define
as senescence. Okay? I think there’s a result that’s
kind of floating around, which is also consistent with
that. That is, we all know the wonderful experiment
of expressing telomerase in human fibroblasts which
causes immortalization. It doesn’t do that in mouse
cells, which also argues that the length of telomeres
in mouse cells is not what is triggering this growth

DR. NABEL: John, I am going to just end

with one last question. You referred to the notion of
program of senescence and it being dominant. I am
just wondering, has anyone ever done a cell fusion
experiment where you have taken cells approaching
their limit and then fused to neo-natal cells. Is it
in fact dominant?
DR. SEDIVY: Yes. Those are very old and classical experiments. In general of course, senescence is a dominant state.

DR. NABEL: Thanks. The last talk in this session is from Frits Fallaux. The title of his talk is using defined adenoviral genes and primary human cells for the generation of immortalized cell substrates.

DR. FALLAUX: First of all, I would like to thank your organization for inviting me here. The subject of my talk will be on the generation and characterization of new helper cell lines for the construction, provocation, and protection of recominence replication effective adenoviral vectors. In the past few years, the interest in vectors derived from human viruses. This is caused by the fact that from the many years of intensive fundamental research on human adenoviruses, it has been found that adenoviruses have several favorable
characteristics, including high stability of variance. The variance is very easy to grow into pure with very high fibers. It has a very broad host range. Importantly, it has the capacity to transduce non-mitotic cells. This makes adenovirus a very potent gene therapy. It is known that it has very low kinisity, and there is ample experience with adenoviruses as vaccines. This slide shows a schematic representation of the adenoviral genome. It is a double stranded linear DNA molecular of approximately 36 KD, carrying several genes, flanked by inverted herminal repeats. The genes are sub-divided in so-called early genes and in late genes, depending whether they are stressed early or late during the lytic infection. This slide shows you a scheme of the classical methods to construct the common
adenoviruses. All currently used adenoviruses carry a deletion in E-1. This renders the virus replication effective, and it also provides space to insert therapeutic genes.

Now in the old days, we used to isolate the DNA from wild type adenoviruses at 5 or S-2. In purified DNA, and I just—the restriction enzyme cla-1, which puts ones in area region one. We then purify the large fragment.

In addition, it also needs the construction of an adaptor plasmid which carries the transcriptase unit, including geno-fenchfras, but also the left inverted herminal repeat and a part of the adenovirus sequence which is also present in the large fragment. Pro-construction of these two molecules in so-called helper cells, and the helper cell is the 293 cell made by Frank Reim. Upon close inspection, another mination occurs, creating now the recombinant adenovirus, carrying the gene of interest at the
position that we want.

You can proficate these elongated viruses due to the fact that the helper cells complement the missing elong function.

Now despite the encouraging results of things so far with the use of recombinant adenoviruses, there are also several problems associated with the use of such vectors. These problems include the growth infectivity range. That is, you do not only infect the target cells, but also non-target cells. This may cause pathogenicity.

Also, the viruses are rather immunogenic.

We only leave off E-1, and all the other viral genes are still present and can be expressed to low levels, resulting in numerous responses by the host, both humoral responses, antibodies, and cellular responses against new cells. The cells are killed and the therapeutic effect is lost within several weeks.

Another issue is the occurrence of replication competent adenovirus, abbreviated RCA. I
will focus on this topic. There are various sources of replication competent adenoviruses. In a sort of infection, during the production of viral, or as an earlier stage, or during the construction of the recombinant factor, especially when you use classical methods, if you use the large clavon fragments. If the digestion if not complete, you have RCA, namely the wild virus.

It has also been shown recently that you can generate RCA by homologous recrimination because the factor and the helper cell carry adenovirus sequences that overlap. As a result, by homologous recrimination, you can get RCA. I will focus on this source of RCA.

Well, how does it work, homologous recrimination resulting in RCA? This is a scheme of a typical elongated factor. This is a scheme of the integrated adenovirus sequences in the helper cells. The helper cell line is 293, and more
recently, we made alternative cell line 911. Both helper cells carry the evon A, evon B in chorion regions. But in addition, they also carry sequences that enclose structural protein lines, downstream of evon B, and upstream of evon A, be left for determinal repeat. Those sequences are also present in the factors. So that a sequence overlap 5 prime and 3 prime of the therapeutic gene. As a result, you can get homologous recrimination by which the recominance virus now trades its therapeutic gene for region E-1, and becomes replication competent. Now what you can do about this is to avoid the sequence overlap. We’ll come back to that later. Well there are only a few helper cell lines available when you work with recombinant adenoviruses. We are fortunately in our lab to have three of them, including the two in our free cell line, recently an iomosa line, and even more recently,
the PER cell line. All three cell lines are obviously of human origin. They are all derived from primary diploid embryonic cells. 293 is derived from kidney cells, 911 from retinoblasts, and PER C-6 as well.

Now when I started to work with recombinant adenoviruses in our lab, which is the lab of Professor van der Eb at the Leiden University, I used obviously 293 cells, and I met with some technical difficulties. Since we had a panel of adenovirus transformed human cells, including cells of kidney, lung, and retinoblasts, I decided to screen a panel of cells in order to find an alternative for 293.

From this panel of cells, I selected one particular retinal cell line. We named it cell line 911. The reason for this name was to get the attention of our colleagues in the U.S.

(Laughter.)
Well, the construct we used to make the 911 cell line is shown over here. It carries the adenovirus sera type 5 nucleotides 87 to 5,788, including evon A and evon B.

Now I want to be short on the 911 cell line. The most important findings were that they performed very good in virus titrations. We also found that the virus use of 911 are up to three times higher with various viruses, also recombinant viruses, three times higher than obtained from 293.

Some other characteristics of this cell line are that they express very high levels of evon A and evon B, are highly transfecetable, which is important when you want to construct recombinant viruses at the classical method. The use of the viruses are very high, as told, and they perform very well in titration assays. So we concluded that 911 is a good alterative for 293.

However, I have shown you the construct we used to generate the 911 cell line. We now have a situation which is similar to 293. Namely, and also
in 911 cells, besides evon A and evon B encoding sequences, also sequences of the left inverted termin are repeat, and sequences in part encoding protein 9 are present. So again, there is overlap and you can create RCA.

So what we decided to do is start all over again and make now the cell line in combination with a so-called matched vector, now sequence overlap. What we did was to make a so-called packaging construct carrying only the evon A and evon B encoding sequences in which evon A is driven by PGK, a heterologous promoter, and a heterologous poly and signal, and lay matched vectors that are deleted of exactly that elong region which is present in the packaging construct. Thus affording sequence overlap and thus eliminating homologous recrimination as a source of RCA.

This shows you one of the packaging
constructs we constructed. Present are adenovirus sera 5, sequences four, five line to 3,511. Those are only the evon A and evon B encoding sequences. Evon A is driven by the human PGK promoter. Evon B is under its natural promoter, and directly flanking the evon B stop codon as the polyadenylation signal derived from hepatitis B virus.

Now before we decided to transfect in this vection construct into our retinoblasts, we decided to do some functional assays with this construct first.

We did this because first we only had a few frozen ampoules of the retinoblasts. Second, the packaging construct contains several PCR fragments. This slide shows you some of the functions or features of adenovirus evon A. The features are that domains 1 and 2 are involved in the regulation of expression of genes. Evon A is known to associate at the protein level with cellular proteins, P-105 RB, cyclin A, P-300. I think the list is growing. All
these different features result in the transformation
and immortalization.

Not shown is the feature or function of

evon B. Evon B prevents the cells from growing into
apoptosis as a result of the activities of evon A.

Now this is the actual functional assay we
performed. At the left is shown the constructs we
tested. At the right is shown the number of colonies
we obtained upon transfection of one or five microprin
of these plasmids into baby rat kidney cells. When we
transfected in a construct expressing only evon A, we
only found on average one focus or colony upon
transfection of one microgram. This is very low.

This is caused by the fact that expression of evon A
in primary cells, in the absence of evon B, is toxic,
caus[ing] apyltosis.

You can see this in the second line.

Transfected this plasmid to come in plasmid in evon B,
and we did obtain reasonable amounts of foci.
Obviously also the construct we used to generate 911 resulted in focus formation.

Unfortunately, also when you are packaging construct, which we used to make the PER cells gave foci, thus indicating that the packaging construct allowed the functional expression of early region non-probenes.

So then we went to the actual experiment, transfected the packaging construct into primary human diploid retinalblasts. We could establish seven clonal cell lines. We tested these clones for first, expression of evon A and evon B proteins.

Now we found that all clones expressed very high levels of evon A in both 55 and 21 K evon B, when compared to 293 and 911. We also looked at vector use. We looked at three clones, clone 3, 5, and 6. As you can see, we found that the three PER clones tested exhibited similar use of recombinant viruses, compared to 293 and 911. Since PER C6 played the highest use, we decided to analyze this clone in further detail.
Now of course the major issue for us was to test whether or not our approach to use the PER cells in combination with matched adenovirus vectors would reduce or even eliminate the generation of RCA. This testing has been performed at the enzyme. What I did was to amplify an RCA free master stock of a typical adenovirus vector and amplify it to 293 or PER C6. What I found, I can summarize it for you, is that amplification of 293 resulted in RCA positive vector batches in approximately 50 percent of the places.

Now for a clinical setting, that means that you might consider to throw away half of what you had made. In the case of PER C6, fortunately in none of the batches amplified on PER C6, we were able to detect RCA, not even in a large scale production setting.

So we concluded that our strategy to make a PER cell in combination with new matched vectors
severely reduced, maybe even eliminated the RCA problem, at least by homologous recrimination.

The next two slides summarize some of the other features of PER C6 cells that contain three to five copies of the packaging construct, very high levels of evon A and evon B, comparable to 293 and 911. Good use of the different vectors, also similar to the other two producer cells.

The cell line was very stable. We have now come over passage 250 actually. So far we have not detected RCA, and the list of productions with different vectors is still increasing.

We have a master cell bank available for PER C6, also a working cell bank. Importantly, the PER cells were made on a GLP conditions, using certified U.S. bovine serum and trypsin. Currently, InterGene is doing all kinds of tests which were necessary for the use in the chemical setting, including mycoplasma and sterility testing. In the
12 academic lab, you can simply draw the cells to
13 standard medium.
14 Finally, I would like to thank all the
15 people that are involved in this project. Number one,
16 the PER cells were generated in the lab of Professor
17 van der Eb at Leiden University, in the Applied
18 Virology Group, supervised by Dr. Gugen. The
19 packaging constructs were made by Edie von Frel of
20 InterGene, and all the downstream processing, a lot of
21 work is currently being performed by InterGene,
22 supervised by Valeria in the Adenovirus group,
23 supervised by Dr. von Laud. As I told, all the RCA
24 testing was performed at Genzyme by Kathy Hay here.
25 Thank you.

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1 (Applause.)

2 DR. NABEL: Thank you. We’ll take
3 questions.
4 DR. FRIED: Mike Fried. Do these cells
5 form tumor in nude mice?
DR. FALLAUX: Actually we did not test that yet. However, we did test this for the 911 cell line, the weakly tumorogen in nude mice. So you might expect the PER cells would exhibit the same feature with respect to that.

DR. FRIED: What is weakly tumorogen?

DR. FALLAUX: Compared to some of the let’s say adenovirus sera type 12 transformed rodent cells, they are much less tumorigenic.

DR. SHEETS: Becky Sheets, FDA. I had a similar question, but I have a couple of other. Does the PER cell stay diploid or is it aneuploid? Also, the individual from whom you obtained the retina, did they have wild type RB genes or were they—you know, was this someone that died of retinal blastoma?

DR. FALLAUX: For your first question, they are no longer diploid. I don’t know all the details, but we do find chromosome duplications I guess in PER C6 because we have tested recently.

Your second question. This may sound
silly, but we are currently trying to find out the

information of the donor. The cells were isolated in
the early 1980s, and we’re now working backwards to
find out those details.

DR. NABEL: John?

AUDIENCE MEMBER: 293 cells have become
quite popular in the laboratory for reasons that have
little to do with their ability to support adenovirus
vector replication. Have you checked these other
cells, for example, high levels of transvectibility in
being a good host for other kinds of viruses and that
kind of thing that make 293 cells so beloved by many
virologists?

DR. FALLAUX: In fact, all adenovirus
transformed cells are as highly transvectible as 293
cells.

DR. NABEL: Okay. If there are no further
questions, then I think we can just proceed onto the
panel discussion. John Coffin will chair that. If
the panel members want to come forward and get
started.

DR. COFFIN: By my calculation, we are
running almost exactly an hour late. A check with the
board outside reveals that we really probably can not
go much past 3:00 before we start to lose people quite

seriously. So we probably should shoot for an hour in
which we either have a lot or a little to do,
depending on the will of the crowd and our host.

This panel discussion actually as two
functions. One is a discussion of the last topic
covered. That is the designer cell substrates, two
talks we heard today and the one yesterday. Then
secondly, where we really earn the generous honoraria
that FDA is paying us, where we try to summarize and
hopefully answer some questions that might be useful
to our host in terms of development of policy, ideas
for further meetings, experimentation and so on.

Tentatively we’ll plan to sort of split
the discussion half and half between these issues, but
I think we can play that by ear as we go along.

Again, I expect widespread audience participation,
particularly since these are topics that I myself am
not really actively working in and am familiar with.
The questions that on the first part, on

the designer cell substrates, that we were charged to
address are summarized on this overhead. Before I
turn it on, I want to apologize in advance for two

things. One is my handwriting is very bad, so you are
going to be subjected to that for a while. Secondly,
I was given a rather blunt instrument to write with.

Thirdly, of course I’m not well enough organized like

some of the previous chairs who have prepared these
ahead of time.

So this is a paraphrase, I hope an
accurate one, of the issues that were raised, that are
raised in the points to consider. The first regarding
designer cell substrates is the issue of whether cells
that are derived by the kinds of defined means that we have seen, and we have seen the example of introduction of telomerase plus or minus oncogenes, viral oncogenes, or viral oncogenes alone whose function among other things was to stimulate or inactivate genes that are involved in senescence. Whether cells that are created in this way in fact offer significant safety, create safety issues relative to other cell lines, whether they offer advantages or disadvantages, whether we can go through the sort of defined risk algorithm that was given to us at the beginning of the meeting, to address these and anything else.

So if we could get onto the first point here. Are there significant safety issues relative to tumor or neoplastic cell lines? In other words, uncharacterized, what we should call it perhaps, uncharacterized cell lines. Cell lines that have just been handed to us either in tumors or that have arisen
by means we don’t really understand very much about in
culture.

Would anybody on the panel care to --

DR. SEDIVY: Yes. I would just like to

make a very brief comment about designer cells. I was
asked to talk about the history of immortalization.
In fact, what my lab works on is more related to
interventions, genetic interventions in human cells.
So you know, obviously we have talked about putting an
H tert and putting in various viral oncogenes. So
really here the issue is can we make a cell line that
is immortal and it has a particular spectrum of
phenotypes that we want by absolutely defined genetic
interventions. I think the answer now is yes. We can
do a lot better than putting in SV-40 large T or E-1A
or E-7, because as have heard even today, we don’t
really know exactly down to the last T what these
viral proteins are doing to the cell.

So in fact, what we can do is we can
delete cellular genes using gene knockouts, and
produce very much the same effect. That is, we can
really now contemplate really designing cells without
the use of viral oncogenes. I think that putting an
H tert is obviously a necessary step, but this is a
cellular gene. So I think that’s probably okay.

DR. ONIONS: It’s really a question of
clarification from my point of view. But it seems to
me that one of the advantages of cell lines that come
from potential oncogenic background is they have some
of the features from mass culture that industrity.
That is, they can be grown in an anchorage-independent
way. They can be grown in high density in fermenters.

What’s the position with telomerase immortalized?
I assume actually they are mimicking much
more the kinds of cell type that Dr. Hayflick would
define as—I forgot what he used, apologies—the
first stage of cell strains. That probably do not
have those particular phenotypic properties. Do you
know what the stage of those cells are?
DR. SEDIVY: Well, you know, we have a really limited experience. This game has only been played for a few months, maybe a year in some privileged labs. I think what we’re really talking about here is proof of principle. In my lab, we’re not interested in growing cells in fermenters. We are interested in cell cycle progression. But I think if somebody wanted to make a cell line that grows well in fermenters, I think it may be a good idea to contemplate some of these new approaches.

DR. ONIONS: Yes. The point I was trying to make was that if you start down the road of trying to produce new cells and immortalize them, and you try and use procedures that you think are of in a sense, the safest, whatever that means, those might not be the steps that you actually need to actually produce an industrially useable cell line regardless of the importance of the science. The practical end may not
be what you want.

DR. FRIED: What else besides, you said you would knock out genes? I mean would this be in human cell lines or instead of using the viral proteins?

DR. SEDIVY: I think in general, the first thing you have to do is you have to immortalize the cell. For that reason, we put an HH tert. That is a technical feature that we need because to do the knockouts, we have—you know, there’s limited time to do a knockout. We can do two knockouts before a human fibroblast undergoes senescence. But if we want to do more, and we’re obviously interested in doing a lot more, it is very convenient to put tert in there at that point.

If you don’t want tert, you know, tert now comes flanked with lock sites, so that you can take it out later on if you are interested in that.

DR. COFFIN: Can you be more specific
about what you would knock out?

DR. SEDIVY: Pardon me?

DR. COFFIN: Want you to be more specific about what you are knocking out. Which genes have you knocked out?

DR. SEDIVY: Well what we are interested in doing is we are interested in dissecting the machinery that establishes senescence. So not in a single cell line, but at this point, we have P-21, cip1, RB, B-53, and P-16 ink 4A knockouts in various combinations. So you know, the vectors are available.

One interesting point about human cells that is interesting to the technocrats I think is that until this time, we do not see the requirement for isogeneic DNA. So in fact, these vectors that we have made, and some other labs have contributed to this, can be used and essentially—and have been used in any human cell with equivalent frequency of recombination.

DR. HAYFLICK: Yes. We have a lot of
information about the biological properties of H tert transformed normal human cells now. There are the ones with which Choma is familiar, are now as I indicated several days ago, approaching, and in one or two cases beyond 400 PDLs. Many of them are beginning to show some signs of aneuploidy. They are still anchorage dependent. Their virus spectrum seems to be identical to that with which we are familiar prior to H tert transformation. Finally, the studies that have been done on animal inoculation of these cells indicates that they are not neoplastic. So that we do know that much.

However, we are not talking about two other classes of cell populations that are important candidates to consider. I mentioned these in my opening remarks on Tuesday night. Namely, normal human diploid cells that can be immortalized using repeated treatments of exposure to chemical carcinogens. We published on this on a population
that explanation defines about 25 years ago.

It’s called SUSM-1. It’s freely available. It is from a normal human diploid fibroblast transformed by multiple exposures to MMNG.

A second class of cells that also falls into this category of normal human cell populations transformed with something other than a virus, by way of example, is KMSD-6 transformed by a former student of mine, using multiple exposures to cobalt 60 radiation. That cell population was also described and in fact is used commercially today. It was described about 25 years ago.

I think it is very important to realize that it’s possible to immortalize well-characterized normal human diploid cells like WI38 and MRC5 and perhaps others, utilizing non-viral means.

I would also like to repeat what I mentioned the other day because it’s been restated again, and it’s not accurate. That is, that there are
spontaneously transformed normal human cell populations. I will be happy to provide the references to whoever would like to have them.

DR. COFFIN: Any response to that? Do we feel in terms of this first issue that there are significant safety, differential safety issues of these kind of cells relative to the relatively uncharacterized lines?

DR. MINOR: From the point of view of the infectious agent side of things, I mean I don’t see much difference between, you know, a brand new tumorigenic cell line that appears in your hand and one that’s actually being designed to actually appear like that. It seems to me they are both uncharacterized and you would have to look at both of them very carefully. I’m not sure that you have new infectious issues simply because you designed it to be transformed.

DR. FRIED: Something came up the first
day. Would it be worth getting new cells that really
grow well with defined media so we can avoid serum and
any problems that come with that as something one
might think about?

DR. COFFIN: If you avoid serum, I think
would be a highly desirable trait in vaccine
production if one could engineer that. Obviously
there are BSE issues, and that sort of raises what
might be the tip of an iceberg. Is that a practical
goal for production?

DR. FALLAUX: Actually, for the
production, we now can grow PER C6 in serum-free media
in suspension.

MR. LEWIS: Lewis, FDA. Are there any
restrictions to the telomerase immortalization? In
other words, there are some populations that you can
immortalize and others that you can’t, or is this a
universal way of immortalizing all types of human
cells?

DR. SEDIVY: You know, I am by no means
expert on this, so I can, you know, basically restate what I think is already out in the literature. That is that you can definitely immortalize human fibroblasts, pre-senescence fibroblasts by putting H tert in. That’s been shown now in a number of laboratories.

You can also immortalize retinal pigmented epithelial cells as reported by the Texas group. I am not sure whether it’s been published yet, but I have heard that you can immortalize T cells, CD8 positive peripheral lymphocytes. Jim McDougall says that you can not immortalize keratinocytes unless he said something different yesterday, in that you need to interfere with the RB pathway in addition to putting H tert. There’s also some indication that breast epithelial cells may need an additional step to become immortalized. That’s all I know at this time.

DR. LEWIS: Since you can immortalize T
cells and you suspect that those would grow in suspension like normal T cells, and you could grow them in any large suspension culture, that you would need to.

DR. SEDIVY: I don’t think this work has been published, so I think I’m just telling you something that I heard at another meeting. So maybe we should just cool it. But I would presume that if they grew in suspension before tert, they would grow afterwards as well.

DR. COFFIN: Obviously these immortalized T cells would be of great interest to people who are interested in growing attenuated HIV vaccine.

DR. FRIED: But I mean once you have something that’s immortalized, you could always select for something that grows in suspension, or you could try anyway.

DR. COFFIN: But when doing that, of course as soon as you start selecting for these
13 additional characteristics, when you are introducing 
14 new and uncharacterized genetic changes.
15                DR. FRIED:  Right.
16                DR. COFFIN:  That these are less important 
17 than safety issues, than changes that might have led 
18 to immortalization in the first place.
19                DR. SEDIVY:  You know, I think I would 
20 also like to second the point that was brought up 
21 earlier.  That is that sure, we can always make the 
22 claim that we know exactly what we did to the cells 
23 ourselves, but we don’t know what the cells have done 
24 on their own during those zillions of passages that 
25 they are growing in my lab.  It’s definitely being 
224
1 documented that H tert immortalized fibroblasts are 
2 karyotypically very stable.  But if you passage them 
3 for long periods of time, you will find anemploy 
4 these.  So I don’t think that that’s any different 
5 from any other established cell lines.
6                So it’s really, you know what you did, but
DR. COFFIN: But you don’t know what happened. To bring a point to this, if one is concerned about issues of what might happen with DNA from the cells that was carried along, then it sounds like, it sounds from what I’m hearing like there may not be a great deal of difference between using these cells and using these kinds of cells as compared to using relatively uncharacterized cells.

Although there are very good reasons for making such cell lines, that this particular issue may not be the most important one.

DR. SEDIVY: You know, you could do some pretty neat things that under certain circumstances may be very advantageous. For example, if somebody wanted to knock out the endogenous PR gene, that could be done. You could make a cell line like that.

DR. COFFIN: You can also use factors that make these cell lines highly susceptible to viruses
you might want to be growing on them, and things like that.

AUDIENCE MEMBER: I’ll just make a comment on one of the papilloma transform cell lines that Margaret Stanley initially isolated. It harbors—it was from a cervical dysplasia and harbors episomal HPV-16 from which E-6 and E-7 are expressed.

Paul Lambert sub-cloned an isolate that carried generous, approximately a thousand copies of this episome in a fairly homogenous state. The problem with it, and it sounds very appealing to have episomal maintenance of your E-7 gene. The problem is it’s dreadfully unstable, and nobody has been able to keep it with the episome. It tends to integrate and completely rearrange, and has gone aneuploid.

So any efforts toward trying to utilize an episome are probably doomed to a degree of failure because of the risk of—well, you have to maintain episomal replication in addition to your chromosomal replication. It just doesn’t seem to work.
DR. COFFIN: And this of course, this kind of issue gets far more amplified when we’re talking about growing up 10 to the 13th.

AUDIENCE MEMBER: Yes, absolutely. We can’t keep this thing going for three months.

DR. FRIED: Also, every time you knock out an allele, you have to lock out the other alleles to get them both. So that means more passages, and they get away. I don’t know whether that’s good or bad. I mean if you finally end up with the cell type that has a lot of positive features, it may not really matter whether they are instability of chromosomal, and stable or rearranged.

DR. ONIONS: Just as a general principle about whether it’s useful or not to engineer cells rather than go out and select a transformed cell, a pre-existing transformed cell from a tumor, it does strike me that again, that it’s under control and that you have a number of choices.
The kinds of studies that PER C-6 has been involved in give you a very precise engineered system that’s absolutely ideal for vector production. But it perhaps also highlights with respect to the mistake that was made. That is, that you had another possibility here where you could actually choose the cell. You could validate its origin. You could check the person. That is the other advantage of you being able to engineer materials, that you can actually pre-select the actual source of the material that you start with. That would have been an advantage that was unfortunately missed in this particular case.

That’s not to undervalue the value of these cells, but it does seem to me that that’s what engineering cells can give you. It gives you control at each stage of the process.

DR. FALLAUX: Can I mention that it’s nowadays rather difficult to take primary human, especially immuno material.
DR. ONIONS: It was—I understand only too well. It’s not at the end of the day a criticism of PER C6 success, which I think are excellent. But really just that where possible, that that should be done.

DR. COFFIN: Okay. So I think we have a consensus here that there’s lots of useful things about such cells, but that we really don’t know whether they enhance any particular safety issue or not. I think that’s a sort of at least some sort of closure on that particular point.

The points we were asked to address also included the use of the defined risk algorithm that was mentioned at the beginning of the meeting, to evaluate these kinds of things. Andy Lewis had that on his slide, which I have asked him to put back on. This will also, I mean with this, we will sort of segway into the general discussion as well I
think, because these are the issues.

So the question is, can we go through and
do this, and is it possible in this particular case,
just using this as an example of this kind of

procedure, to assess the level of risk posed by these
issues, infectivity, infectious and so on,
quantitatively. My own feeling right now is that

we’re no where near a position to do this, certainly
for DNA issues. We might be able to put some sort of
numbers on viral issues. It’s a little hard to see
exactly how because there’s so many different ones,

which could have a different contribution. But maybe
we can get some further comments on these sort of
issues from the panel.

DR. ONIONS: My only comment, and I
understand why a defined risk approach was used, and
it certainly makes you think. I mean that’s one of
its great virtues. I think one of the real intrinsic

problems, that if you applied, you can give yourself
a false sense of security. It would strike me that
Phil’s story about SV40, if it turns out that is the
origin, SV40 in people, would have given you such a false sense of security, I think, because you might have come through that exercise in the 1960s. I’m not sure how you would have predicted that that agent was there a priori.

So I’m not sure that you can give guarantees that 1 and 10 to the $6^{th}$ dose is one-half X, if you don’t know what X you are looking for.

DR. SEDIVY: Yes. I mean I broadly agree with that. I think it’s worth trying to do some sort of numerical calculations, so long as you don’t believe the numbers that you get out at the end of it.

(Laughter.) Because I think one thing it will do is it will tell you where you think you are confident, on what stage of the process you are actually confident. Then you can actually question whether your confidence is misplaced or not. But I think if you come out with
a number, I think you are asking for trouble.

DR. LEWIS: Yes. I don’t think that we discounted that. I think in sort of going through this thing, what we were trying to do was to figure

out exactly where we can be reasonably confident of what we’re doing and where we can not be confident of what we’re doing. But we always recognize that when push comes down to shove, the bottom line is that we’ll always—we can never be sure.

So at some point in time, it requires a leap of faith to say this product or this cell line,

or whatever, can in fact be used. I think what we’re trying to figure out a way to do is to make sure that when we get to the point or we have to make that leap of faith, that it’s better to find than it would be if we did nothing at all.

So the attempt here is to develop sort of a way of thinking about narrowing that margin of error, or at least to develop a margin of error that
is better than it would be if we’re just doing it on
intuition.

DR. ONIONS: I think that’s absolutely
right. I was taken by Neil Cashman’s risk assessment,
quantitative risk assessment today. I think again,
what it did, although I think he himself didn’t
believe certain the numbers at the end of the day, it
makes you think about the process. I think that’s
fine and I think I would agree with that.

DR. HUGHES: One of the things that’s true
about the numbers that I’ve seen is no one has
attempted to put what I guess I would call a
confidence interval on the numbers. One of the things
that makes me uncomfortable is that I think in some
cases the uncertainty is as large as the number. I
would feel a bit more comfortable with a calculation

with which I’m fundamentally uncomfortable, if I had
a better notion of how uncertain people were about the
assumptions they were making in generating the numbers
in the first place.

DR. ONIONS: I think that’s what we’re all saying, is I think I started it off by criticizing the whole approach. I think what Phil said is what I think. I think Dr. Lewis said the same thing. That is, don’t believe the numbers. All it is is gives you a manner of approaching what are the issues, really. I think that’s the way it should be treated. I agree. I don’t think anyone should believe the numbers at the end of the day.

DR. HUGHES: I think it might help if when someone put down a number, they at least put down a range of numbers, and that would generate a range of confidence at the end. I think what people will see when they do that, is that the ability to define the confidence interval is going to expand when you multiply the numbers together. I think that act may in a sense help define how uncertain the number actually is.

DR. COFFIN: I think from a regulatory
standpoint, what often happens is that the far end of
the confidence interval on the worst possible side is
taken, and then that’s propagated through. You never
see the other side.

DR. ONIONS: I don’t want to just go into
an academic discussion about risk assessment because
I’m not really interested in it, in that formal sense,
but there are two other approaches that are used. The
ingeniusry industry uses a form of analysis that
doesn’t do risk assessment like that. It actually
looks for holes. It looks for what could go wrong.
In a sense, that’s really probably what we ought to be
doing. Then there are four mechanisms of that kind of
analysis.

The other form of analysis is the one that
has become fashionable in the U.K., which is this
concept of the precautionary principle, which
ultimately, it seems to me, means you don’t ever do
anything because you never know what might happen,
which seems to me completely dumb.

DR. COFFIN: So we’re voting against the precautionary principle. You can’t be sure of anything, but you can be sure of that.

Are there any other points anybody would like to make about this? One could say that this is a useful way to organize your thoughts on this subject, but shouldn’t be taken as giving you either additional grounds or comfort or discouragement, unless you actually had a situation where you had measurable quantities.

Are there any other issues or questions regarding the designer cell substrate issue that anybody wishes to raise?

DR. SHEETS: Hi. Becky Sheets, FDA. I am going to ask the whole panel what I tried to ask Dr. Hughes earlier. That is, the kinds of questions that sponsors ask us. One question I would ask is we’ve heard a lot of people in this meeting say that the
oncogenic DNA issue has been put to bed. Has it?

DR. COFFIN: That segways us into the next, into the general discussion, which is fine.

Before we go into that, can we see if there are any other specific issues regarding cell substrates?

DR. SHEETS: Any questions about the quantitative?

DR. COFFIN: If we do that, we can turn off that slide, put up my next one, and then you can ask the question.

DR. SHEETS: There’s one question.

AUDIENCE MEMBER: Jerry Sato from Merck.

I understand the reluctance to put a number on something when you have such degree of lack of confidence and the assumptions that are going into it.

But I do think that getting an order of magnitude of where we are is actually helpful in our thinking about what we feel comfortable going forward with or not.
When you have a lack of confidence in each of those areas, you also have to ask the question, what are the chances that all of your assumptions are wrong? In other words, are two of them wrong, three of them wrong, five of them wrong, seven of them wrong? Because you have to put a degree of that’s not likely to happen. So if you multiply the lower end of the confidence interval for all those things, then you will never do anything. But that’s not the way it works in reality.

So I think it would be useful for somebody from the engineering community, where they design bridges that aren’t supposed to fall down and other things, to try and put a bit little more sophistication into this analysis, because in the end, somebody is going to ask our community, which is the regulatory community, the academic community, and the industrial community, for the number or at least what they thought the number was when they went forward with their act of faith. Because there is a certain
amount of common sense that goes into it, which is the basis of the act of faith. Then there is whatever kind of quantitation we can put into it. It’s the combination of those two things that I think we are going to have to reassure the general public about.

DR. HUGHES: I would recommend to you the book Strategy and Conscience by Anatol Rappaport, which attempts to deal with the issues having to do with what was called strategic thinking, in which you calculate, for example, the probability of some unlikely event, such as thermonuclear war. Mr. Rappaport does a very good job of making clear why doing the calculations when you don’t have the proper data is in fact a very risky and misleading proposition.

AUDIENCE MEMBER: I guess it might be worth pointing out that there are, however, some cases where you clearly do have the proper data. Right?

You know the sensitivity of a specific assay for a
specific adventitious agent, if in fact you choose to figure out what it is.

So you can actually answer, based on those kinds of questions, and based on that kind of data, the specific question of how sure are you that something isn’t there. If a better assay comes along, and it’s still negative, then you can say by how much more certain you are.

So just because you can not come up with good estimates for some of the numbers, seems to me it would be crazy to throw the baby out with the bath water and claim then that you shouldn’t attempt to come up with good numbers for those things that you can.

DR. RABINOVICH: Gina Rabinovich, NIH. A non-regulator asking a question from experience learned this summer, in which we have been dealing with using a quantitative number, i.e. the numbers that the Federal agencies and the global agencies has
set for acceptable limits of mercury, i.e. methyl
mercury, and then trying to attempt to understand what
those uncertainty factors mean for thimerosal and
vaccine exposure im.
The concern I have, and I think it has
been heard, is that these numbers take on a life of
their own. They become the standard against which
things are measured. So that that kind of concern
needs to be entered into, attempting to use the data
when those data do exist, but understanding the limits
to it.

DR. COFFIN: That’s inherent in

regulation, that things become standard.

DR. LEWIS: Lewis, FDA. To follow up a
little bit on what Phil was saying. I think one of
the areas that we could approach with some confidence
is the ones who saw Keith Peden’s data last night
using the tac man assay to detect JC, BK, and SV50 in
human tissues.
Now if someone comes in with substrates they derive from a neuroblastoma, which we learned at the DNA tumor virus meeting a year or so ago, is it’s usually contaminated with BK virus. We wanted to be sure that there was no BK virus in that substrate.

Then we could apply this assay with a fairly sufficient level of sophistication and say with some certainty, based on the volumes and things that were tested, the level at which that particular genome or that particular virus is absent. So I think that we sort of view that as a possible starting point for a quantitative approach.

Now obviously you can’t do that unless you know exactly the probes and things that you are working with, and you define the limits of their ability to detect things. But I think this is one of the sort of examples that was going through our mind when we were thinking about how to do this.

So you start at the place where you might
be able to generate some relevant data that’s meaningful. The other stuff will fall into place as we get better.

DR. ONIONS: I mean I think that’s exactly right. I think more and more that we move to assays, that we get good quantification on, we can validate them, and it’s the sensitivity and limits of detection. I think all of that is absolutely essential. I mean I absolutely 100 percent concur with that. I think it does add confidence to those specific questions.

I think when you are asking specific questions, then I 100 percent agree. I think my concern is perhaps that where you try and make assumptions, for instance, of residual DNA. I mean I was the one who said I thought it should be put to bed. That is because all the evidence that I had heard didn’t convince me that there was a risk.

On the other hand, I actually believe that no one has done the right experiment to actually
convince me of that, in a formal scientific sense. So we’re then dealing with the area of conjecture. That conjecture is based on non-quantitative data.

DR. COFFIN: We’re leading into you, Becky.

DR. SHEETS: I’m patient.

DR. COOK: I’m sorry. Jim Cook. As I was sitting there thinking about how you would describe these issues to a patient or to a group who is asking you about the wisdom of using a vaccine, it seems like in addition to trying to generate some logic about calculations of numbers and risks, that every opportunity that you have, it would be worthwhile going back to history and saying well, we have done virtually something like this along the way ever since vaccines have been developed, and the experience with this approach has been the following. So maybe there could be some real numbers in a historical sense, used to color or give some more
real meaning to these, what are otherwise theoretical things, to help communicate this to the public, as well as to provide some, a little bit more logic than just phenomenology to the calculations that are being made now. So if history is used to color the estimations, that might be of some use.

DR. COFFIN: How comforting is it to tell patients that there are three or four cases of paralytic polio?

DR. COOK: Say it again?

DR. COFFIN: How comforting is it to tell

patients that there are three or four cases of

paralytic polio?

DR. COOK: Well, I think you have to be honest with them. You say look, you are one of 250 million people. The odds for your child is the following, and I think it’s a very good idea to use this vaccine.

It is those kinds of conversations that
eventually lead adolescents into getting hepatitis B vaccine. We’re doing a miserable job of this, by the way. I think as a culture, you know, if we bat 50 percent, we’re lucky.

DR. COFFIN: That’s clear in light of things in the movies, because lately we’re doing a terrible job where these people show up with these anecdotal cases of somebody’s child gets vaccinated, and then two months later is diagnosed with autism. It’s automatically due to the vaccine.

DR. ONIONS: But I think history can also be a dangerous thing. I mean the British government has been criticized, partly justifiably, but I think partly unfairly for the problems of the way BSE problem goes up.

But the issues concerning public health were based on people were asked what is the risk, what is the risk of the human population of the BSE outbreak, when we had a few hundred cases of BSE in
cattle. Well, there were only a few hundred cases.

The general assumption was, and it was a widely shared
general assumption by those who were informing the
area, the people who worked on scrapie and so on,
well, scrapie has no evidence whatsoever of scrapie
transmission to man. We have been eating scrapie-
infected sheep for generations and it doesn’t seem to
have been transmitted to people. There is no evidence
of that whatsoever.

The probable likelihood that BSE is of
scrapie origin that’s gone through the rendering
process, because we have never seen a spontaneous
spongiform encephalopathy in cattle, so that’s
probable origin ipso facto, you know, there’s no
problem.

It wasn’t quite as glib as that because
actually within a year, all the controls on human food
were in place and so on and so forth. They were badly
conducted, but they were theoretically in place.

So I think history can also be dangerous.
I’m not sure that we can always learn the right lesson.

AUDIENCE MEMBER: I get your point, but I think there is a difference between an ongoing epidemic that’s yet resolved, and the experience of X number of hundreds of millions of doses of polio vaccine. So what I am saying is, if you are going to make a calculation about the likelihood of one in a million or one in 10 or 100 million happening with a vero cell or with a primary cell, you can say well, we’ve got experience making polio vaccine in vero cells, and there have been the following hundreds of million doses given, and the likelihood is that when the incidence of the real disease gets so low that you are finally going to see some background. That’s the real --

DR. ONIONS: I’m sorry. You misunderstand. What you are saying, I absolutely
agree.

DR. COFFIN: Do you want to continue this discussion or do you want to break into --

AUDIENCE MEMBER: Very similar aspect, although a little bit less scientific. As I perceive the discussion, of course there’s this highly sophisticated, highly conscientious scientific community, and there’s the general public on the other side.

The general public, to my perception,

consists of at least two sub-groups. One group that is sort of generally benevolent and would believe scientists. But there is a very strong group that is not believing scientists. They are sort of using that as a political tool to attract attention. In our country, we have had this experience with the Green Party, that has become very influential in the European Union and maybe in other countries as well.

Now one thing I also, since we’re ending
and coming to the end of this meeting, would like to raise, isn’t it the responsibility of scientists also to do something to better educate the public? I know this is a utopian goal, but at least if we could increase enough of people in the general public who are educated or better educated in science and biology and biomedic issues, we would at least have a political community that might support scientific issues more valuable than we have had it so far.

You know, in our country at least, every time when the Greens demanded to stop all biomedical, all gene technology research, and it course never happened. After they come to power, maybe they have different outlooks on life. But I think as a scientific community, unless we do something at least for the future, we might be in a very difficult situation to defend certain issues.

If I confronted some of the violent ideologically pure Greens in our country, because the
trick is, we have been discussing here, I’m sure they

would say “Shut it down because this is unsafe,
totally unsafe.”

So what I am trying to recommend is we

have to do something to have more people in the
general public who can appraise and can assess the
difficulty and the uncertainty in any biological
research. We can never get down this figure to 10 to

the minus 80. So we have to raise understanding on

the other side.

DR. SHEETS: So have we put oncogenic DNA
to bed?

DR. COFFIN: No. I would like to use the
sort of summary—I think that’s in a sense, an
overhanging issue. We have talked about infectious

risks and measurements and so on considerably today
and in the past few days. I think an overhanging
issue is this oncogenic DNA issue regarding the

specific charge of the meeting, which is the use of
tumor versus other kinds of cells, tumor and
neoplastic whatever, transformed cells versus other kinds of cells as substrates for vaccine production.

Although I think many of us here, perhaps all that’s here, feel this is not a risk to be really concerned about in a scientific sense, I think many of us here might agree that the issue is not completely put to bed in the sense that we can’t put any real good numbers on it.

So now if you ask your question.

DR. SHEETS: Okay. Has oncogenic DNA been put to bed?

DR. HUGHES: I will answer in two different ways. I will give you my opinion, personal opinion, and then I will tell you what I think should be done, which is slightly different.

Personally, and this would apply if you approached me to do something to myself, I am not concerned. However, it is my view that the data that we have, particularly for the consequences of putting
DNA into animals, is not sufficient to satisfy me as a scientist. I am going to try to help my colleague, Dr. Coffin, and some of my colleagues at the FDA to try and organize a simple study that would be more satisfying to me.

I think I would feel more comfortable if we had more data that was of the experiments done on a larger scale under more controlled conditions. I think that would give me a greater degree of comfort.

DR. ONIONS: I think I almost entirely concur with that comment actually. I did say a comment, and said it partly to be provocative, but on the other hand, I think I share that opinion. I have seen nothing that would convince me at the moment on the data, which there’s a singular lack of, or just from I suppose theoretical reasoning, to suggest that this would really be a significant danger. But on the other hand, there are the tools now, and I say yet again, but I think some of the
transgenic models offer that possibility for testing, whether or not DNA is a risk. It can be done in a series of graded experiments from taking the worst case examples of actually simply just repeating—of injecting oncogenes at various titrations into animals that are already primed with oncogenes as a transgenic, down to taking tumor DNA down to taking normal DNA. I mean those experiments, they are pretty straight forward to do. I mean the interpretation might be a bit more complex, but they can be done. I think they are worth doing. It might help you to put some limits, broad limits on the thing we’re all just making conjectures about.

DR. MINOR: I don’t think it’s been put to bed either.

DR. COFFIN: For the same general reason?

DR. MINOR: Broadly speaking. I mean I think it’s clearly a very, very complex issue about
how you actually induce a tumor. So you do your 3T3 assays and you pick up H ras. Okay? I mean it’s an artifact of 3T3s or was that just a question of how common H ras is.

If you go and put your DNA in intravenously, is that the same as putting it in subcutaneously, for example? I mean if you put it in because it’s been picked up by an envelope virus, is that going to make any—will it be picked up by an envelope virus? Will that make any difference? I mean it seems to me that there are so many sort of loose ends to it that I don’t think while there is no evidence that DNA is tumorigenic, and I buy that 100 percent, it doesn’t seem to me that it’s necessarily been dealt with properly. That is why I guess I am agreeing with what the previous two speakers said.

DR. LOEWER: So as I already have said, I personally believe that there’s not a real big risk
with purely oncogenated DNA. Purely oncogenated means three or five or six. But I realized that there is still, I believe, lack of experimental data. This was already mentioned by John Coffin, in saying that since 18 years, this question is on the table. Since 18 years, no additional experiments have been performed. I would like to propose that regulatory authorities, which are involved in regulation of these biologicals, the major ones, that we should sit together and to join the efforts and maybe decide on experiments which can be done in the foreseeable timeframe. But I look forward to see what types of experiments John may recommend. DR. FRIED: I think most of the evidence we have so far, which is limited, says at least putting DNA into animals, we haven’t seen anything happen. We have only seen in NIH3T3 cells, and we now know that there is a defense mechanism in the cell
when it sees an oncogene. That is this P, this arf, which is the alternative reading frame of P-16. So it’s like an immune system of the cell, specifically for oncogenes. The radiation activation of P-53 is a different pathway. This turns on, and arf activates P-53, and P-53 then closes itself to go through apoptosis.

The only positive things are in NIH3T3 cells. They are the classic cell where the arf gene is inactivated. Probably that’s why people have been using that for years. They are very easy to transfect because maybe even transfection kills the cells in terms of P-53.

But that said, I would like to see a lot more injection of DNA from different tumor lines into animals, and to really put it to bed.

DR. COFFIN: It’s striking to me that one of the very few, if only successful experiments with injected activated oncogene DNA in chickens is the one
that Hsing-Jien Kung did that was off-site, or in any
animal, that was Hsing-Jien Kung. It was actually
fairly efficient sort of transient transformation of
cells, but transformation of cells is always
transient. It’s virtually impossible to immortalize
them. They are much harder then human cells to
immortalize. It’s been done once, to my knowledge.
So there may be something very
fundamentally different going on in that model,
because there may be some fundamental difference in
chickens as compared to mice, as compared to humans,
which already have important --

DR. EGAN: Bill Egan from FDA. I would
certainly like to work with you and other regulatory
authorities to try and design and our colleagues in
PHS, to try and design these experiments and do these
experiments, and get the data, and get away from the
remark I quoted from Maurice Hilleman from 30 some-odd
years ago about this debate being a philosophical or
ecclesiastic debate because we simply don’t have the data. Here we are 30 some-odd years later, you know, with the same question, with the same debate. It’s still opinion.

I must say I also feel that personally, myself, I don’t think there is a large risk from the DNA. But then again, the kinds of risks that we’re talking about are very small risks, very, very rare events. Things like one in a million are not acceptable or in many cases are not acceptable. Those are hard data to get.

While I may not feel there is a risk to me, the bottom line basically for the approval of almost any of these vaccines, is would I put this into my children. There it becomes a much more conservative process. If putting it into my children is putting it into other people’s children, it’s the same thing.
DR. HUGHES: If you want one more piece of data that should give you some comfort, it is the experience attempting to make antibody to the oncogene sarc, which involved putting an avian virus into a number of mammalian species under circumstances in which the virus absolutely does not replicate. In adult animals, to my knowledge, no cell growth was ever seen. The only experiments that succeeded, to my knowledge, were those initiated by John Burge, in which he put enormous amounts of ras sarcoma virus of a subgroup that would infect mammalian cells. We are talking sort of $10^{10}$ infectious units. Into immunologically naive baby bunnies. In those animals very transiently, there were small nodules which regressed. In those animals, you did see antibody to the oncogene sarc, implying that there was transient uptake of the DNA, at least probably permanent uptake of the DNA. But even under those circumstances in which the delivery of the DNA is extremely efficient and every copy of the DNA carries a known potent viral
oncogene under circumstances where the DNA will not
replicate but will insert, do you see any permanent
transformation? Again, I’m not suggesting that this

is sufficient. I am one of the advocates of more
experimentation. But the data that we have suggests
that this is not a simple process.

DR. EGAN: No, but I mean these are the
kind of data that start to put brackets around the

numbers for the levels of risk.

DR. COFFIN: Of course you have enormous
problems, including the fact that sarc is never seen.

It’s a human oncogene. For many years, the most
popular viral model.

AUDIENCE MEMBER: I had a question. If
given the unknowns, and given the data that was

presented about hit and run DNA modification
potentials, would the panel in the context of this
type of vaccine development, and given the unknowns,
give the vaccine to someone with a strong family
history of malignancy or who was a cancer survivor who
we know is at increased risk for a second cancer, if
that was you or your family member?

DR. COFFIN: The question, to sort of
focus that a little bit, the question is whether we
would consider there to be a greater risk in certain
sub-populations who might have sort of pre-activated
oncogenes or some other fact of predisposing.

DR. ONIONS: I understand the question,

but it sort of arose -- I will go backwards, because
one of the things that used to concern me was when
people were doing clinical trials with rusvel vectors,
and they were using marker studies. That sort of
study did worry me, because actually what you were
there doing was putting something, inserting something
into somebody who probably already had a preexisting
oncogenic hit. That struck me as being dubious,

mildly.
In this situation, I would have thought that unless you have got somebody with a Li-Fraumeni syndrome or something, that you are dealing with changes that are somatic changes in a few cells, even if they are going to risk of a second cancer. So the likelihood that you hit the right cell is pretty low. So I wouldn’t have thought it up the risk—I’m sure the risk has increased, but I wouldn’t have thought that risk has increased significantly, unless of that sort where you’ve got mutation.

DR. MINOR: It would also depend on what you are trying to protect them against too, wouldn’t it?

DR. SHEETS: Before we lose our entire audience, I wanted to—I think we have gotten a pretty clear answer on the oncogenic DNA issue. I wanted to ask another kind of question that FDA has asked, before we lose our entire panel. That is, are
there risks, additional risks that one perceives in using a continuous cell line such as vero cells, particularly vero cells, which is immortal but not tumorigenic at the levels that vaccines are made, past the level that vaccines are made. Is that worse in any way than diploid cells, for a live viral vaccine?

DR. MINOR: This is apart from the DNA issue?

DR. SHEETS: Well, in a continuous cell line, certainly there are—it may be aneuploid, but it’s not tumorigenic in animals. So you can comment if you’d like about whether you think the DNA is oncogenic.

DR. MINOR: I would say that the DNA from vero is as questionable as the DNA from anything else. I mean John, with whom we discussed these matters, more or less said the same thing. I think that it doesn’t matter how malignant it is. Maybe it depends on how many oncogenes you put in there. So I would have thought that a vero is as questionable as
anything else, or is not as questionable as anything else.

DR. SHEETS: So you wouldn’t suggest to make a live viral vaccine in vero cells?

DR. MINOR: I think it would depend on the live viral vaccine. I mean I think OPV clearly has been made in vero cells. You can scrub it clean. I think you can more or less destroy anything that’s actually hanging on the end.

I have more serious thoughts perhaps about things like a paramixovirus vaccine, because you couldn’t clean it up so much perhaps.

DR. SHEETS: What about the sort of crude, less purified live viral vaccines, not the purified vaccines like we heard about last night with OPV, but the things that are just filtered cell culture supernatant?

DR. MINOR: Right. They might be figit. I’ll tell you that. Which is not to say there’s any
good reason for me to be uncomfortable with them.

It’s just that they make me feel uncomfortable.

DR. COFFIN: We are very fast losing our audience, so I think --

DR. HUGHES: Isn’t it partly the question do you know the life history of your cells as opposed to the state of the cells at the end?

DR. SHEETS: Vero cells are a bank that is well characterized. The reason for the question is that we have numerous live viral vaccines of the sort I described that are being proposed to be made in vero cells. Manufacturers prefer vero cells because one, they can be characterized. Two, you get a high yield.

Three, they can be grown in the sorts of fermenter culture that you heard about.

DR. HUGHES: I’m not particularly bothered as long as I know that the sort of life history of the cell. But I think the question is, if you have a cell that’s been in culture for a long time and has had a
complicated culture history, do you know that history?

AUDIENCE MEMBER: I asked the last panel the same question. It comes down to the question really is the adventitious agent issue put to bed as well. Do we now have the assays in place that can easily be applied to validate the freedom from adventitious agents of these kinds of new cell lines?

The answer that Dr. Broker gave in the last panel suggested that one could attempt to use DNA chips and things like that, which to my knowledge aren’t assays that at least tomorrow I could go out and do on a cell line and give me some confidence.

So my question to you is, sort of using the standard assays that you are all aware of, without developing further assays for this specific purpose,

do we have enough information to be sure that these kinds of new cell lines are safe from the adventitious agent perspective?

DR. ONIONS: That’s another unanswerable
question, isn’t it. I would just make the point that

I think you have to adopt somewhere between good

science and pragmatism. I mean you could

theoretically go and do representation difference

analysis on all these cell lines. Actually, I don’t

think it’s possible because you don’t usually have the

partner. But theoretically you could do that. That’s

not really a practical solution.

It does seem to me that we do know virus
types that tend to be latent in cells, and that it’s

sensible to perhaps think of strategies of widening

the brief of detecting those agents, because I’m not

convinced that the kind of routine types of infecting

-- infectability assays when they work are as

sensitive as PCR, as just Phil pointed out. But I am

not convinced that always the right infectability

assay is present to actually detect certain agents.

So that you are probably relying on a combination of

things. Perhaps we do need to look at redundant PCR

for certain agents.
DR. COFFIN: I would think the producers would have a big attraction, is set up the same assay and use it for everything.

DR. MINOR: I mean I think you could also argue that you have used these assays for looking at human diploid cells and primary cultures, and all that sort of stuff. Right? What’s the difference in principle in terms of adventitious agent contamination between those and the cells you are looking at here? I am not sure there’s much difference. But are the concerns as big or as little.

AUDIENCE MEMBER: For one, I would like to thank Dr. Andy Lewis for bringing us together. This has been a very stimulating week. Last night Dr. Vyas showed us a picture of the thinker. But that actually prompted me to remember that Rodin placed that gentleman directly above the gates of hell.

(Laughter.)

The question I would like to pose right
20 now is whether we’re walking in through the gates or
21 out.

22 DR. COFFIN: I would like to also second

23 the thanks to the organizers for setting this up and
24 bringing us here.

25 DR. LEWIS: Yes. On behalf of the

26 sponsors and those of us at CBER who worked on this,

27 we really appreciate the effort that the session
28 chairs, the panel chairs, and the speakers have put
29 into this meeting. When you attempt to put something
30 like this together, there’s always a question of how
31 it’s going to turn out. I think the success that we
32 have enjoyed here the past three days is a tribute to
33 the work, an incredible amount of work, that has gone
34 on on a very short period of time.
35 I think that I was very concerned when we
36 were trying to contact folks in May to do this by
37 September. For those of you who rose to the
38 challenge, I can’t thank you enough on behalf of the
sponsors.

With that in mind, I hope everybody has a great trip home. Get your papers in whenever you can.

Thank you.

(Whereupon, at 2:57 p.m., the proceedings were concluded.)