

EVOLVING SCIENTIFIC AND REGULATORY PERSPECTIVES ON CELL SUBSTRATES
FOR VACCINE DEVELOPMENT WORKSHOP

On 1/30/07, AJC <ac_aj@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). Gardasil 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>

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U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
FOOD AND DRUG ADMINISTRATION
CENTER FOR BIOLOGICS EVALUATION AND RESEARCH
INTERNATIONAL ASSOCIATION FOR BIOLOGICALS
NATIONAL INSTITUTE OF ALLERGY AND
INFECTIOUS DISEASES
NATIONAL VACCINE PROGRAM OFFICE
WORLD HEALTH ORGANIZATION

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EVOLVING SCIENTIFIC AND REGULATORY PERSPECTIVES ON
CELL SUBSTRATES FOR VACCINE DEVELOPMENT

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WORKSHOP

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Friday,

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.

2

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
Johannes Loewer, M.D.	Panelist

Also Present:

Gary Nabel

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- 1 P-R-O-C-E-E-D-I-N-G-S
- 2 8:04 A.M.
- 3 CHAIRPERSON RABINOVICH: Good morning. I
- 4 would like to welcome you back to Session 6,
- 5 Adventitious Viral Agents in Cell Substrates, and

6 congratulate all those that were here until 10:00 last
7 night for the latest part of the show, including Mr.
8 Harris, who put in a grueling 18-hour day in
9 yesterday, our visual aides person.

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

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

20 What I am going to do is to review firstly
21 all of biologicals, if you like, from an adventitious
22 agent point of view. So it won't just be vaccines.
23 In particular, I will be talking about the range of
24 source materials that people have used in preparing
25 biologicals. There will be a clear message that comes

1 out of that, which is that the more you use well-

2 characterized cells, the better.

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

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

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

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

19 You can use your whole animal as a

20 substrate for growth. I will discuss that in the
21 context of things like influenza vaccines and the
22 like.

23 You can grow material on primary cells.
24 This was the main starting point for things like polio
25 vaccines in the early days, where the SV40 issue

6

1 arose. Finally, you can grow materials on well-

2 characterized cell preparation.

3 The further down the list you go on this
4 thing, probably the happier you are from the
5 adventitious agent point of view.

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

9 Most of these will be human rather than anything else,
10 but really an awful lot of the serious adventitious
11 agent problems that have arisen have arisen because of
12 materials sourced from whole animals or using pooled
13 preparation.

14 The first one on this list here is CJD,
15 Creutzfeldt Jakob Disease, which was transmitted by
16 growth hormone. The growth hormone was produced from
17 human cadaveric material. A very unpleasant disease.
18 It's almost impossible to detect the agent other than
19 by standing back and waiting for the incubation period
20 to go.

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

7

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

6 Scrapie was first shown to be a
7 transmissible agent by the use of a TBE vaccine, which

8 was grown in the brains of sheep. TBE being tick-
9 borne encephalitis, which then transmitted scrapie to
10 a large number of the sheep that were actually
11 inoculated with it. So again, this is a whole animal
12 source material, if you like, that had quite serious
13 consequences, especially if you were a sheep.

14 Over the last 15 or 20 years or so, one of
15 the best examples of serious or disease-causing
16 transmissions of infectious agents has been through
17 human blood and blood-derived materials, clotting
18 factors in particular. In all of these things, the
19 entire alphabet soup of hepatitis viruses has been
20 transmitted by blood product.

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

8

1 using factor 8 to treat hemophilia. I am not sure

2 that that is an acceptable way of actually doing
3 things any more. I am sure that hemophiliacs would
4 agree with that. B-19, paravirus B-19 is still
5 transmitted by clotting factors.

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

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

18 So most of the serious consequences really
19 come from whole animal source materials, if you like.
20 You can use whole animals as substrates. I'm using
21 the term "whole animals" in a fairly broad sense.

22 Eggs in the definition of the Animal Regulated Use Act
23 in the United Kingdom count as an animal because they
24 are embryonated.

25 For many years, rabies vaccines were

9

1 produced in mouse brain or sheep brain. They have
2 quite serious consequences, but not necessarily
3 associated with adventitial agents. You can get
4 encephalitis as a result of immune responses to the
5 non-invasive protein.

6 The Japanese encephalitis vaccine, which
7 is used for travelers in the United Kingdom, is still
8 made in mouse brain. So it's not an unusual source of
9 material, if you like. Smallpox for a long time was
10 made on the scarified flanks of calves. Like I said,
11 isn't any more. However, while these things seem
12 really quite primitive, in terms of how you make
13 vaccines nowadays, you still have a number of vaccines
14 that are made in eggs. Yellow fever is the classic
15 example, and influenza.

16 Yellow fever is not required to be grown
17 in avian leukosis-free eggs. The reason for that is
18 that there are a number of sites at which it was
19 manufactured throughout the world, where yellow fever
20 is a really very serious problem, such as Nigeria, for
21 example, South America, whatever, where SPF eggs,
22 avian leukosis-free eggs even, were really not freely
23 available. So yellow fever can in principle at least
24 be made in avian leukosis containing eggs, and in fact
25 is. I think there's no evidence that this has an

10

1 adverse consequence. But on the other hand, you
2 wouldn't necessarily want to have a virus in there
3 that you didn't know about.

4 Influenza is an actuated vaccine. Again,
5 it's not made on SPF eggs, that is, specified
6 pathogen-free eggs. They are avian leukosis free, but
7 they are not free of all the other variety of
8 pathogens that you would choose to screen for measles

9 vaccine production system, for example.

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

17 Primary cultures as been described
18 previously around here, are really cultures that are
19 made directly from the animal. So they are not one
20 pass. They are directly from the animal, if you like.
21 Here are a number of examples where agents are
22 actually being found or at least located in these kind
23 of cultures.

24 SV40 is one that I'm going to talk about
25 in some detail in a minute. This was in polio

1 vaccines in the 1950s and very early 1960s, probably,
2 a source from rhesus monkey kidney. Polio vaccines

3 are still made on monkey kidney, though they are not
4 usually on rhesus monkey kidney. It would be
5 cynomologous or something like that, for reasons which
6 I'll describe in a moment.

7 Nonetheless, a great deal of vaccine is
8 still made in primary monkey kidney cells. There are
9 reasons for that. There's a deep conservatism I think
10 about changing the vaccine production process if you
11 have a vaccine that works, largely because you are
12 dealing with a prophylactic material rather than a
13 therapeutic material. So you don't want to mess about
14 with anything if it's reasonably safe and effective.

15 I'll mention very briefly the defective
16 retrovirus story in chick embryos. I think Jim
17 Robertson will probably mention this in more detail,
18 but I will mention that just as I go by. Finally
19 recently, the FDA released a talk paper on a
20 preparation of urokinase, which is used in treating
21 the heart. This material was grown from primary
22 cultures made from aborted fetuses. I think it was

23 aborted fetuses or miscarriages, or whatever. There
24 were quite a variety of infectious agents were
25 actually found in this. I believe this one has now

12

1 been suspended.

2 The point is that there are still a large
3 number of materials which are made on really quite
4 basic culture systems, if you like, where adventitious
5 agents are a serious consideration, if you like. So

6 it's not all continuous cell lines versus the rest.
7 I mean there are—most of the vaccines that are made
8 in the world probably come from other primary cultures

9 or eggs or things of that nature.

10 I will now talk about SV40. I'm sure in
11 this audience there are people who know far more about
12 SV40 than I do. But nonetheless, I'll talk about this

13 from what you might call the regulatory adventitious
14 agent point of view, if you like.

15 So it's a very common polyoma virus of old

16 world monkeys, and particularly rhesus macaques. The

17 difficulty with this was that when the rhesus macaque
18 monkeys are sacrificed and a primary monkey kidney
19 culture is made from him or her, as the case may be,
20 a silent infection is set up. So there is on evidence
21 of infection just by looking at the cultures. In
22 fact, these cultures can throw out as much SV40 as
23 they do polio, when you start infecting it with polio.
24 So you wind up with a culture that's just stiff with
25 adventitious agent which you really don't want.

13

1 It's able to transform non simian cells in
2 vitro, and it can be tumorigenic if you have the right
3 kind of animal that you put it into. Between 55 and
4 62, probably at least a third of all the vaccines that
5 were made on these kind of cultures, because they were
6 pooled and the like, were almost certainly
7 contaminated with SV40. It wasn't a trivial
8 contamination. It was really quite a serious
9 contamination.
10 Because it was mainly an activated polio

11 vaccine, there wouldn't have been that much live SV40
12 in it perhaps. But SV40 is more resistant to formalin
13 than polio is. So almost everybody who received the
14 shot of inactivated polio in the 1950s, which would
15 include me, would have received live SV40 in some form
16 or another.

17 So the concern is really summarized here,
18 which is basically that everybody, I mean this is my
19 own take on it, that everybody—I mean you can argue
20 that it might not have been sort of everybody, but I
21 think it probably was. But almost everybody who
22 received the full course of polio vaccine between 1955
23 and 1965, also got live SV40 stuck into them. That's
24 millions of people basically.

25 There were epidemiological studies that

14

1 were done at the time which really didn't cause much
2 concern, but they can all be criticized. Some of the
3 studies were really quite short-term, about two or
4 three years or so, looking to see if there were cancer

5 effects basically, as a result of SV40. It may be
6 that two or three years is not enough to actually find
7 such an effect, if it actually exists.

8 The longest which was assumed was over a
9 period of about 19 years. Most of the individuals
10 involved in that study would have been oral polio
11 vaccine recipients rather than inactivated polio
12 vaccine recipients. So they have had it by mouth
13 rather than by injection. Again, you could argue that
14 that might not be the right cohort to actually be
15 looking at.

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

23 However, in 1992, Michaili Carboni and
24 colleagues and others, a number of others, including

25 Janet Butelle down in Texas and the like, identified

15

1 SV40 sequences which were present in a variety of
2 relatively rare tumors. So mesothelioma, which is the
3 asbestos tumor, osteosarcomas, meningiomas, actually
4 the young choroid plexus tumors of children, these
5 sequences do appear to be genuine SV40 sequences.

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

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

18 Now SV40 was discovered around 1961 or

19 1962 or thereabouts, 1960 perhaps. Directly it was
20 discouraged. There were precautions put in place to
21 exclude it from polio vaccines, because it was known
22 to be a tumor kind of virus, if you like. These were
23 the kind of things that were put in place. They are
24 listed in WHO requirements from about 1962 onwards.
25 They reached their final fully flowered form, if you

16

1 like, by about 1965. A number of countries certainly
2 had put this in place before that.

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

9 The second thing is, you remember I said
10 that it was the rhesus macaques with the problems.
11 The problem was that the cell cultures didn't show any
12 sign of having defect, when they were actually

13 infected with SV40. What you can do then is you can
14 use species, such as cynomolgus or patus monkeys,
15 where the primary monkey kidney culture cell, when
16 infected with SV40, will actually wrinkle up and die
17 on you. So at least you know you have got something
18 nasty and you can throw it away.

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

23 Around the period that this was taking
24 place, wild caught monkeys were being used extensively
25 in vaccine production. Up to a half of the cultures

17

1 would have been thrown away because of adventitious
2 agent contamination, mainly foamy virus, but certainly
3 other things as well. I think that just illustrates
4 the kind of lack of control, if you like, over the
5 source materials that was going on, and the extent to
6 which adventitious agents are really a serious problem

7 in finding monkey kidney cultures or primary cultures
8 in general.

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

13 Nonetheless, a significant, if indeed not
14 a large proportion of the world's supply of polio
15 vaccine is still made on primary monkey kidney cells,
16 which should really fit this kind of criteria for
17 excluding SV40.

18 One of the questions that then arises is
19 were these precautions good enough? What we did at
20 NIBSC, because we happened to have about 150-odd
21 batches of vaccine archived from the years, was to go
22 back and look at them by PCR. PCR of course is the
23 cat's pajamas. It's really the best technique that
24 anybody ever invented in terms of sensitivity. It's
25 probably about as good as infectivity, at least in our

1 hands anyway.

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

10 In fact, the only preparation which had
11 any SV40 sequence in it at all was a seed virus which
12 was used by a manufacturer for making vaccine from.

13 The amount that was in there was around two logs worth
14 of genome as opposed to seven logs of genomes in a
15 really full-fledged infected preparation. So there
16 wasn't that much in there. The manufacturer had also
17 treated this stuff with toluidine blue, which is
18 supposed to kill of SV40. This was done on the advice
19 of Albert Sabin back in 1960-something or other.

20 But nonetheless, it does seem to me that

21 it's rather a foolish thing to have a seed that's got
22 SV40 sequences in it at all. I think the WHO
23 requirements have now been changed so the seed has to
24 be checked to see if it does have SV40 sequences in
25 it or not.

19

1 This particular seed was not infectious
2 SV40. We did some quite serious studies on it, like
3 transfecting the DNA into cells to see if it would
4 work, infecting monkeys with it to see if we could
5 actually get seroconversion. There was no
6 seroconversion. So there was no infectious virus
7 there that we could actually detect. But nonetheless,
8 the seed did have material in it.

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

14 So our conclusion from that was then that

15 really as soon as these kind of precautions were put
16 in place, no SV40 would have been present in all polio
17 vaccines used, at least in the United Kingdom and I
18 would guess in the United States as well, because it's
19 after the same kind of precautions were put in place.

20 So the precautions were adequate. Which means that
21 SV40 exposure of the population through polio vaccines
22 would have stopped around 1962.

23 So what you then have is the problem of
24 the chorioid plexus and appendinoma tumors, which
25 occur in children who are around two years of age or

20

1 maybe less. You have to say well how did they get a
2 hold of the SV40 sequences? One possibility, which is
3 mooted with some enthusiasm is that maybe you are
4 getting passage of SV40 from parents who did receive
5 the SV40 contaminated polio vaccine to their children.

6 So how this stuff gets around is quite important.

7 One of the things that we have been
8 involved in is doing serological surveys of

9 populations to see who has got SV40 antibodies and who
10 hasn't. It is about a five percent seropositivity by
11 the assay that we're using at least. It seems to peak
12 at around age 10 or thereabouts, and doesn't arise
13 after that.

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

23 We have access to a number of sinomorgous
24 breeding colonies. One of them at least is absolutely
25 riddled with SV40. It's chronically infected. They

1 are all infected basically.

2 So this is just four examples of this
3 particular colony. There's about another 50 or so.
4 This happens all the time. The mothers here are
5 highly sera positive to SV40, all of them. What
6 happens is that the mother and the baby stay together
7 for about six months until the baby is weaned. Then
8 the babies are taken off, no longer being babies of
9 course. They are all banged up together in one
10 gigantic sort of teenage squabbling colony.

11 At the time of weaning, the babies are
12 uniformly negative. So despite the fact they have
13 been on the mother for six months, they have not sera
14 converted to SV40. Almost immediately you bang them
15 up together, or at least within about a month or so,
16 they sera convert. So we actually have a sera
17 conversion panel here, if you like, with about 50 or
18 100 or so sera, where the babies actually were
19 seronegative and then become seropositive.

20 My view on this is probably that the
21 babies don't get infected until you bang them up
22 together. But it may be that they are infected, but

23 they are just not seropositive. So what we have to do
24 here is to fish out the virus from these animals here,
25 and see if it looks like the mother's virus or if it

22

1 looks like the other babies' virus.

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

9 One last quick thing or two last slides
10 here. One is about reverse transcriptase of vaccines.
11 Dr. Schuepbach will be talking later and Jim Robertson
12 will be talking in a moment about detection of reverse
13 transcriptase in chicken cell grown vaccines, such as
14 flu or yellow fever or measles, mumps, rubella.
15 This appears to be due to the presence of

16 defective non-infectious particles. There are
17 sequences from EAV and ALV both in these things, a
18 ratio of about nine to one as I understand it. It
19 does seem to me that you are not really quite sure

20 what the AV sequence is in there and what ALV sequence
21 is in there. It's probably going to vary from chicken
22 to chicken in so far as these chickens have not been

23 bred. In other words, every egg is a new experiment.
24 You are really not quite sure what you are dealing
25 with in that. I think that is quite an unfortunate

23

1 position to be in. I'm not sure how you control it.

2 Finally, this is my last slide, and this
3 has to do with characterized cells. The issues that
4 I have been dealing with really have been to do with
5 primary cells and primary cell problems where the

6 virus comes in direct from the animal origin. I think
7 there is no doubt in my mind that that's the main
8 source of concern in terms of human health.

9 Nonetheless, there are clearly problems

10 which also arise with characterized cells and the
11 continuous cell lines, in particular. We have some
12 down here.

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

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

24 There are examples of things like BVDV
25 contaminating cells which are growing in culture, and

24

1 also other bovine viruses contaminating cells in
2 culture, particularly when they are grown on a very
3 large scale. Whether or not that poses a hazard is

4 another matter, but clearly there must be methods in
5 place to actually detect them.

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

16 This is a question of actually getting the
17 cells infected while they are actually burning in
18 culture. So while family cells are clearly a major
19 problem, and while whole animal sources, if you like,
20 are probably the biggest hazard which is likely to be
21 raised in terms of human health, biological aspects to
22 do with well characterized cell banks, where viruses
23 may be introduced from biological materials or they

24 may be introduced by mice walking across the top of
25 the fermenter or whatever, are nonetheless a

25

1 significant matter. It really is not totally clear

2 whether these things have an implication for human
3 health. But I think you would be wise to make sure
4 that they are not actually present.

5 That's where I stop. Thank you.

6 (Applause.)

7 CHAIRPERSON RABINOVICH: Please identify
8 yourself.

9 DR. COFFIN: John Coffin of Tufts. That
10 was a really nice summary actually, Phil. But some
11 caution might be called for in translating the results
12 of vertical transmission experiments from monkeys to
13 humans.

14 As far as we know, simian immuno
15 deficiency virus in monkey populations are not
16 transmitted vertically. Yet HIV-1 is transmitted with
17 reasonable efficiency vertically in human populations.

18 So there may be some underlying biological difference
19 that perhaps a very subtle one, that promotes this
20 kind of transmission in people, where you wouldn't see
21 it necessarily in monkey models.

22 DR. MINOR: Yes. I take your point. We
23 are doing the studies for two reasons. Firstly, to
24 look at natural transmission to monkey on the grounds
25 that it might be a model, although I take your point

26

1 entirely. But also to supply serum conversion panels
2 so that we can try and sort out specificity of
3 immunological reactions as well. I take your point
4 entirely.

5 DR. ONIONS: David Onions, Glasgow.

6 Phil, when people switched to cynomologous
7 monkeys, and I can see the reason because you can pick
8 up SV40. That's very clear. But how do you know that
9 at the same time, you have not invented a new problem,
10 that you have got another polyoma virus in that
11 species that you are not detecting. I mean has anyone

12 done redundant PCR to look?

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

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

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

27

1 anything yet.

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

4 DR. MINOR: Sorry? Say that again.

5 DR. LEWIS: I take that seroconversion is

6 by neutralization?

7 DR. MINOR: That's right. Yes.

8 DR. LEWIS: Have you had any chance to

9 look at monkey breast milk to see how long they may be

10 treating antibody, so that the newborns can be

11 passive immune?

12 DR. MINOR: Right. The answer is no. We

13 have discussed, for example, getting the urine out of

14 these monkeys after they are banged up together. I am

15 told that actually chasing them around the floor is

16 insuperable. I'm not sure you can actually pry a baby

17 monkey from her mother long enough to actually milk

18 her. It's a worthwhile question. I think we'll have

19 another go and see if we can do something about it.

20 There may be some resistance, however.

21 DR. BROKER: Tom Broker, UAB. I wanted to

22 follow up on that exact question. We are facing the

23 same problem with potential vertical transmission of

24 human papilloma viruses. I'll mention it later in my

25 own presentation, but briefly, it does appear there is

1 some protective immunity during nursing. On the other
2 hand, removal of an infant, say through adoption to
3 another family, is the highest risk factor for a child
4 acquiring laryngeal papillomatosis later in life. So
5 a two to three year delay.

6 DR. MINOR: So is the assumption then that
7 the infant is infected, but it's not infected properly
8 then?

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

13 So what I would propose as a potential
14 experiment is to literally take the, if possible, take
15 the baby monkey immediately away from the mother, and
16 don't allow it to nurse, and then just have different
17 lengths of time of nursing to see if this onset of
18 seroconversion is affected by a timing mechanism.
19 Alternatively, don't ever let that baby monkey be

20 housed with other baby monkeys in the daycare center,
21 and keep it with the mother even if it's not nursing,
22 and see if it fails to seroconvert.

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

25 DR. MINOR: That's right.

29

1 DR. BROKER: Or is it receiving a period

2 of important passive protection from the mom.

3 DR. MINOR: I figured we could do that by
4 looking at the actual strain of viruses the monkeys
5 get infected with. We have a number of different gang

6 rooms, if you like. If you get a different strain in
7 each gang room, but it's the same strain within a gang
8 room, then I think that will answer the same question.

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

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

13 I know you didn't have time to expand on it, but I

14 think it is extremely important. That is the need for
15 those organizations who discover a new virus or some

16 contaminant, cell population used for vaccine
17 production or in a production run, to make that
18 publicly known.

19 I think that the declaration by Genentech,

20 who has published this information under their name,
21 that an NBM contamination occurred in a 10,000
22 fermenter is an act of great courage. I think that

23 that kind of courage, this declaration by other
24 companies in this field, is very necessary for the
25 health of this industry.

30

1 I understand from some of the remarks that

2 have been made that there are others that are known to
3 a small coterie of people here that have not been
4 publicly declared. I urge all of you to think about
5 this seriously because it can and will have a great

6 impact on this industry. Thank you.

7 DR. MINOR: I agree totally with that. It

8 does seem to me that sooner or later the information

9 will leak out. I think the industry looks very bad.

10 DR. VAN DER EB: Van der Eb, Leiden. Did

11 I understand it correctly that ferrisfaruses were

12 found in human embryo material that was used for

13 urokinase production?

14 DR. MINOR: I think the FDA can answer

15 this one better than me, yes. But I mean that was my

16 understanding of it. It's out on the net.

17 DR. VAN DER EB: But where does it come

18 from?

19 AUDIENCE MEMBER: I think it's a rea

20 virus.

21 DR. MINOR: It's various rea viruses, plus

22 others.

23 DR. VAN DER EB: I see. Okay.

24 DR. FRIED: Mike Fried. Was any of the

25 old vaccines from the 1960s that were contaminated,

1 were they PCR'd up to show that the virus was the same

2 as being found today? Because it's also possible that
3 we all have a latent SV40 type virus which likes to
4 grow in tumor cells, and that's why you find it. It's
5 a passenger. But I mean since there's polymorphisms
6 in the sequence, if you can go back to the 1960s and
7 then find out if it's the same thing that we find
8 today, it would be helpful.

9 DR. MINOR: We looked at, when you say the
10 1960s, I have to emphasize this is very early 60s.
11 Certainly the things that we have got which came out
12 positive weren't used in the UK, or they might have
13 been used somewhere else.

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

21 CHAIRPERSON RABINOVICH: Last question

22 please.

23 MS. MARCUS: Carole Marcus Sequora from
24 Bassey Consulting.

25 I just wanted to clarify that urokinase is

32

1 produced from cells. It's not aborted fetuses. It's
2 newborns who did not survive. Just for the record.

3 DR. MINOR: Thank you.

4 MS. MARCUS: It was reactivated virus.

5 DR. MINOR: I'm sorry about that.

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

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

16 ultimately focusing down on the vaccine issues.
17 So I will begin with some direct
18 information regarding retrovirus situations with
19 biologicals. I will go onto look at how some of the
20 regulatory guidelines deal with the issue of
21 retroviruses. I will go into look at RTase testing,
22 which is a reasonably current them just now, and
23 finish up looking at the recent situation of the
24 finding of retroviral-like particles in avian cells.
25 So to begin with, here is a short list of

33

1 the incidence of retrovirus contamination found in
2 biologicals in general, not just vaccines. I have
3 sub-divided these into two groups here. You see this
4 upper half here, this is where we have in the past had
5 overt adventitious contamination by a retrovirus of a
6 biological. For instance, being mentioned earlier,
7 ALV, that causes virus in yellow fever vaccine by
8 virtue of producing the vaccine in embryonated eggs

9 infected with the virus. The other one that was
10 mentioned earlier by Phil, HIV and blood products.

11 The bottom half here is a quite, somewhat
12 separate type of contamination. In fact, you might

13 find it equaler to call it contamination or not.

14 Certainly these are not adventitious situations.

15 These are situations in which an endogenous

16 retroviral-like particle is present in the

17 manufacturing process.

18 In the first instance here, it's

19 established that murine hybridomas used in the

20 manufacture of monoclonal antibodies produced,

21 secretes C type particles. These have been tested in

22 a variety of other mammalian, including human cell

23 lines, and generally are not infectious.

24 The titre can be very high for these types

25 of particles. You can get 10 to the sixth particles

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1 per mil. I've even seen 11 particles per mil in one

2 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 interstitial 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

23 contamination? There are a couple of guidelines I
24 would like to bring to your attention. The first one
25 here is an ICH guideline, which looks at viral safety

35

1 evaluation. Admittedly it is only for biotech
2 products. The scope of the guideline does comment
3 that this is not, this guideline is not applicable to
4 vaccines. But I think it is worth looking at what it
5 says about virus contamination.

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

12 The guideline goes on to state what is
13 acceptable and what is not acceptable in the
14 manufacturing process. The only two cases which are
15 generally acceptable of the first two cases, Case A,
16 where you have got no virus, and Case B, where you

17 have got a non-pathogenic retrovirus. The other cases
18 are only exceptional. Generally you don't want one at
19 all. The manufacturing is not allowed when you have
20 got a virus contamination.

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

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1 and these recombinant products are highly purified.

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

6 When it comes to testing for retroviruses,
7 this guideline has several—many other guidelines in
8 the past have indicated, the types of assays being
9 used for retroviruses, specific infectivity assays,
10 electron microscopy and transcriptase assays, are the

11 three general approaches for checking for retrovirus
12 contamination.

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

17 If I can concentrate now on the RTase
18 assays. The traditional type of assay involves
19 incorporating a nucleotide precursor, a labeled
20 precursor of some kind into an assay using a rather
21 synthetic type of template. Then more recently of
22 course we have the PERTs, PB RT, AMP RT type of
23 assays, which includes a PCR amplification step, with
24 the result that these type of assays are incredibly
25 more sensitive than the more, as I can call it,

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1 traditional type of assay, and what is often quoted as
2 up to a million fold times more sensitive by virtue of
3 incorporating a polymerase chain reaction.

4 Now using this type of assay, the cat was

5 set among the pigeons. When this paper came out, I
6 might even say that the fox was set amongst the
7 chicken coop. Detection of reverse transcribed
8 activity in live attenuated virus vaccines. This
9 quite naturally caused a bit of concern as to what was
10 going on here. The vaccines indicated, the one common
11 feature was that ovine produced in eggs of some kind,
12 measles vaccine out of CEF primed cultures, similarly
13 mumps. Yellow fever and influenza in ovo. But not
14 measles vaccine out of human diploid cells or rubella
15 vaccine out of human diploid cells. So the common
16 link here seemed to be the CEF, the chicken source
17 used in the production of the vaccine.

18 We joined in the boat here and started
19 looking at this issue. Every type of hen fluid that
20 we have looked at, CF fluids or an type fluid from a
21 variety of different strains of hen have all been
22 positive in the assay for reverse transcriptase.

23 Summarily, quail, jungle fowl, and pheasants are
24 positive.

25 The types of sources of fluids which have

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1 been negative for reverse transcriptase are listed
2 here. Some species are not positive, turkey and duck
3 cultures, quite a range of human cell lines. Simian
4 rabbits have been tested and found to be negative. So
5 the clear source of this RTase that was being picked
6 up in the vaccines is quite clearly of chicken avian
7 origin.

8 We would want to look at—I should add
9 that this RTase was known at the time to be particles
10 associated and appears in the supernate of the cells.
11 We are going to look at this particle to see if it
12 would pick up any infectivity. In all, we looked at
13 10 different cell lines, mainly human, but including
14 rabbits and turkey. Over 21 tests and 116 passages.

15 In each case, in every test and at every
16 passage level, the cultures were negative for reverse
17 transcriptase activity. There's absolutely no
18 indication that this particle is infectious. Since

19 then, CBER and CDC have also come up with similar
20 data, including use of PBMCs. No infectivity
21 associated with these RTase containing particles.

22 Where might these be coming from?

23 Presumably they are derived from endogenous
24 retroviral-like elements in the chicken. The
25 information to date regarding such elements in the

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1 chicken genome are quite well characterized EV loci,
2 which are related to the avian chosis virus family,
3 and more recently discovered about 10 years ago, EAV-0
4 family, which is an older element than EV, and then
5 older still, ART-CH and CH-1 elements.

6 The information at the time and pretty
7 much where it still exists is that we knew that
8 there's a line of chickens which was negative for EV.

9 It had been eliminated from the genome. This line of
10 chickens, the culture fluids were positive for RTase.
11 So we knew that it had to be at least one of these
12 elements producing RT activity. At the same time, you

13 couldn't eliminate the fact that EV might also be
14 producing RT activity. The best bet was EAV-0, given
15 the sequence information that was present at the time.

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

24 So what can happen here retrovirus-like
25 particles, defective particles being produced from

40

1 endogenous elements both from EV and EAV-0 family of
2 endogenous elements. The presence of the RNA and
3 reverse transcriptase in a particulate fraction leads one
4 to come to the conclusion that we have retroviral like
5 particles in the CF fluids of the chick cells, which
6 is present in the vaccines measles and mumps.

7 The absence of infectivity in the current
8 genetic information, sequence information that we have
9 on EV loci and the EAV-0 family of endogenous elements
10 strongly indicates that these particles are defective
11 viral particles. The only question mark that remains
12 from the regulation point of view, but also scientific
13 point of view, the possibility of pseudotype formation
14 during vaccine manufacture. The current evidence
15 suggests the particles that are defective in the
16 envelope-like protein and so there's a particulate of
17 pseudotype formation with the glycoprotein of vaccine
18 viruses being grown in the CF cells.

19 So to summarize a couple of these issues
20 then, from the practical point of view, testing for
21 reverse transcriptase as an indicator of retroviral
22 contamination, these assays are evolving, changing all
23 the time. One has to take into consideration the
24 strength of the assay and the validity of the assay.
25 There may be different requirements within an assay

1 for different sources of RT. It may be necessary to
2 use some other sort of method to assess the
3 significance of any RT detected because we know that
4 RT activity can derive from other enzymes. Telomerase
5 is or DNA polymerase, cellular DNA polymerase is.
6 These features are not specific to the more recent
7 sensitive type of assays involving PCR, the parents,
8 and the PBRT. These features were also factors that
9 had to be considered in the more traditional types of
10 assays.

11 It is often quoted that the RT levels in
12 chick cells is very low, given that it was detected by
13 a very, very sensitive assay, and has not been
14 detected by the more traditional type of assays.
15 Certainly some preliminary data that I have got
16 suggests that it is not quite as low as we first
17 thought. Really this RT activity in chick cells and
18 ultimately in vaccines is only just below the level of
19 detection of the more traditional type of assay. In
20 fact, this was a relatively novel phenomenon

21 discovered just a few years ago. It was in fact first
22 reported 20 years ago in the late 1970s by Berne and
23 Hofschneider at the Max Planck Institute in Munich.
24 They reported the presence of a novel type of RT
25 enzyme in chick embryos and in chick cells. That was

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1 in the days before PCR.

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

11 So to look at the RT issues from a
12 regulatory point of view, a couple of comments I would
13 make. First, that these are state of the art
14 technologies. When these highly sensitive assays

15 first came about, it posed very useful from a research
16 point of view to what use are they in a routine
17 manufacturing validated type of assay.
18 I think the time has come where yes, you
19 would say that these are state-of-the-art techniques
20 and can be and should be used for detecting the
21 presence of RT in your manufacturing process. However,
22 when it comes to, for example, chick cells, and until
23 we have a greater understanding of what the levels
24 might mean, and until standards are available, there
25 is really—it is difficult to justify any

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1 requirements to perform RT or PBRT assays on systems,
2 and basically here I am talking about chicks, which
3 inevitably will be positive. We know they are going
4 to be positive, that there's no great need to actually
5 require any manufacturer to do these assays. But
6 certainly there is a still a strong requirement to
7 provide evidence for freedom from retrovirus
8 contamination. This will have to derive from other

9 data. Thank you.

10 (Applause.)

11 CHAIRPERSON RABINOVICH: Thank you, Dr.

12 Robertson.

13 Any questions?

14 AUDIENCE MEMBER: Just a comment. For

15 known endogenous avian retroviruses or exogenous avian

16 retroviruses, of the cell lines, of the cells that you

17 tested for infectivity, only the turkey cells would

18 have given a positive result. I would urge for avian-

19 derived—urge the use of those cells, and a PERT

20 assay is a sensitive readout, for detection of perhaps

21 unknown agents in these vaccines, end products, as

22 being the most sensitive, at least for avian-derived.

23 DR. ROBERTSON: Yes, yes. The turkey

24 cells are sensitive for ray, because it's virus, but

25 apparently not for the RTase. I have no idea, duck

1 cells are also negative, but I have no idea if duck

2 cells are susceptible to the --

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

7 DR. ONIONS: David Onions. I really
8 enjoyed that, Jim. I just want to make a comment on
9 your comment about standards. I think as we heard
10 from Keith last night and what we're doing, and I'm
11 sure George is doing too, using the tac man technology
12 where you can actually quantitate the PCR product.
13 Then if you actually do EN counts of virus particles,
14 dilute these out, you can actually quantitate your
15 assay system and actually determine the number of
16 particles you can detect.

17 Now it seems to me that that is a useful
18 kind of standardization, and that you can then relate
19 that to if you like, a consistency of your starting
20 material, in this case the egg.

21 So I think in that case, applying those
22 techniques does have value, because it gives you a

23 kind of lock-to-lock consistency of your materials.
24 So that if something goes out of spec, then perhaps
25 something odd is going on in those materials.

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

10 A large current high level is when your
11 typical infection goes up to 10^6 , I believe,
12 focus forming units. You are only talking about 10^4
13 drop, lower value. So you are not far away. It is
14 going to be difficult. If you have got an overt
15 contamination going up to 10^6 , I think that

16 will be quite clear on a quantitative basis. But once
17 you drop down a bit, it is difficult to say whether
18 you have got an infection or whether it's just
19 background level of endogenous RT-derived activity
20 that you are picking up.

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

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1 in longitudinal --

2 DR. ROBERTSON: Well the reason I am
3 looking over your shoulder, we have Walid Heneine from
4 CDC. I'm not sure if you are going to say something
5 along those lines, but serologically, there is no real
6 evidence for reaction to ALVs. Epidemiologically,
7 when it first came out, we also, not ourselves but
8 epidemiological colleagues, to provide information.
9 There is no evidence again, for any increase in the

10 incidence of childhood cancers since the onset of
11 measles, mumps vaccination.

12 Walid, were you going to say something?

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

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

23 My comment is that the evidence we have so far on
24 those we have studied in a couple of vaccines between
25 in Europe and in the U.S. suggests that those, for

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1 example, ALVs we're dealing with could be defective
2 because they come from loci that have deletions.
3 However, this may not be true for all the contaminants

4 from it we might find in other vaccines, because these
5 contaminants reflect the particles expressed from
6 these loci in the different cell substrates that Phil
7 mentioned in his talk, that the nature of these
8 particles and their phenotypes would vary depending on
9 the presence of the particular loci in these
10 substrates.

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

13 We might or we might not in certain cases.

14 DR. ROBERTSON: Yes.

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

24 one of the vaccines which contains the EAV.

25 So we found that statistically highly

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1 significant the vaccinees which have received the

2 vaccine which have the higher content, about 80 times

3 higher than the other vaccine which we used to use

4 too. That these patients actually had highly

5 significantly elevated antibodies to HIV 1 and 2,

6 although none of these actually became sero positive.

7 The serious bonds was highest in those individuals

8 that also had a history of measles vaccine.

9 In the second study, this was an influenza

10 vaccine where we tested two different brands, a split

11 vaccine and a rather crude vaccine. We also had a

12 response to HIV bond two in the third generation tests

13 in the vaccine which contained more in the crude

14 vaccine, which contained more of the EAV protein, and

15 again those individuals who had a history of yellow

16 fever vaccination had the highest type, the highest

17 increases.

18 We also tested yellow fever vaccinees by
19 PCR, RNA PCR and DNA PCR for EAV-0 sequences. We
20 found one out of 180 individuals in which both these
21 tests were positive as plasma on PBNC. At the moment,
22 we cannot exclude that this was the result of a
23 contamination, but we are working on that. So I think
24 that the matter is actually not as clear as has been
25 presented by others.

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

8 DR. ROBERTSON: At the end of the day, it
9 would have to be an infectivity assay. There are also
10 some antigen, ELISAs for the viral antigen. I don't
11 have experience with those. I don't know the

12 sensitivity of them. Ultimately you are looking at an
13 infectivity assay which can be performed even on chick
14 cells, which are positive for RTase. One could assess
15 for after several passages on chick cells, looking at
16 an increase in RT activity or increase in antigen.

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

21 DR. ROBERTSON: Well, it would have to be
22 an avian retrovirus if one is performing the assay on
23 chick cells. Propagating the material, the test
24 material in chick cells but using either RTase in
25 general or an ELISA specific for ALV test for

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1 increased presence of either RT or for the presence of
2 ALV antigen.

3 CHAIRPERSON RABINOVICH: Dr. Coffin, the
4 last question?

5 DR. COFFIN: Yes. I'd like to actually

6 address Dr. Schuepbach's comment. Did I understand
7 that you were basing your sero assays on the rationale
8 that there might be cross reactivity between ALV and
9 retro viruses in HIV? There is no rational basis for
10 that. There's virtually no amino acid the same
11 between those two viruses, except for some extremely
12 highly—you know, three or four in pol and some
13 other places. Did you actually assay directly for
14 seroreactivity against ALV? It would have been a much
15 more straight-forward experiment.

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

21 CHAIRPERSON RABINOVICH: Thank you, Dr.
22 Robertson.

23 Our next speaker, Dr. Jorg Schuepbach,
24 from the Swiss National Center for Retrovirology.
25 Induction/activation and detection of occult viral

1 agents that are present in mammalian tissues.

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

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

8 So occult viral agents are agents you
9 don't detect or at least do not easily detect. They
10 may include two groups: a group that includes known
11 agents which are present at two low concentrations for
12 easy detection. The reason for these may be latency;
13 The other group consists of unknown agents. Since we
14 do not have good detection methods for these, they may
15 be present at low or also at higher concentration.

16 Viruses known for their latency or various
17 types of the herpes virus, true, they are latent in
18 various types of non-permissive cells such as neurons,
19 B cells, monocytes, PBLs, and others. They are

20 activated from these latent stages by various kinds of
21 stimulation of their host cells by differentiation,
22 agents by the differentiation of precursor cells, to
23 more mature cells. Again, by other activating agents.

24 Other viruses could be considered in
25 addition to the herpes viruses include the adeno

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1 viruses, the adeno-associated virus and the pathyoma
2 and polyoma viruses of which we heard yesterday, and
3 will hear more in a subsequent talk.

4 Regarding the RNA viruses, I might discuss
5 the measles viruses and of course the retroviruses.

6 When we look at the mechanisms by which we
7 can activate these various viruses, it is mostly by
8 activation of their host cells, by cell stimulation,
9 by induction of cell differentiation of these cells,
10 and then by co-cultivation with cells which are
11 permissive for replication.

12 Now since we have different viruses and
13 host cells systems, these methods vary greatly among

14 the different viruses. If you have unknown viruses,
15 you really don't know what to do.

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

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

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1 The principal goal is to provide a virus production
2 system which is free of such agents. It is suggested
3 here the easiest way of achieving this is actually
4 cellular cloning. Because if you have an agent that
5 is present in only a minority of the cells, the
6 chances that you derive a clone that is free of these
7 agents is very high.

8 If by chance you hit an infected cell, the
9 descendants of that cell will all carry along the
10 virus and of course then we come into a situation
11 which makes detection of unknown viruses and also
12 known viruses much easier because either all of the
13 cells will be infected or none at all.
14 So cellular cloning, if we hit an infected
15 cell, has actually a viral gene amplification effect
16 which is comparable to virus induction activation if
17 it's successful. Most importantly, it is a procedure
18 that works for all the latent viruses except
19 endogenous retroviruses, but these are present anyway
20 in all of the cells.
21 So going on to the detection methods for
22 these agents, let's first talk about known viruses.
23 Since all the cells will be infected, we actually do
24 not need the most sensitive procedures. We do not
25 need procedures that detect the single viral copy.

1 What we need is broadly reactive methods which go

2 detect all the different members of a certain virus
3 group.

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

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

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

22 milligram of DNA or respectively RNA.

23 The principle is very simple. We use
24 biotinylated capture probes which bind to these
25 sequences inquest. We isolate these complexes on

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1 coded beads, wash the rest of the DNA away, and them
2 amplify these by PCR with primus which are located
3 outside of this capture probe.

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

12 Now using this method, it's actually very
13 sensitive. You can detect a single copy here of HIV
14 DNA. We still have double positive signal, is about
15 one copy. This serial dilution was done in the proper

16 range here. The fact that in these two, three last
17 dilutions only one of the two duplicates was positive
18 clearly demonstrates that we are in a Poisson
19 distribution. So we can detect the single copy with
20 this method in 100 microgram.

21 DNA, we have actually demonstrated that
22 there's 95 percent probability we can detect three
23 double standard HIV copies in 100 micrograms of DNA.

24 So now going on to the exclusion of
25 unknown viruses, and I will talk about retroviruses

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1 later, we can actually use the same procedures as I
2 have already described previously. We just have to
3 take care that we really have broadly reactive
4 methods. This is true for molecular based tests as
5 well as for the more classical procedures.

6 Now coming to retrovirus detection, of
7 course also of cell cloning, here we have two
8 situations, the exogenous retrovirus may not be

9 present in none of the cells or in all of the cells.
10 The endogenous retroviruses were always present in all
11 of the cells. The known exogenous retroviruses are
12 detectible by tests for conserved sequences. Of
13 course you might also use universal pool primers for
14 unknown retroviruses—because of the endogenous
15 retroviruses. Not all of which, or very few of which
16 are actually harmful.

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

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

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1 unamplified.

2 Now in most instances, this will simply be

3 the cDNA. There are other possibilities as well. You
4 can take any nucleic amplification procedure, not just
5 PCR. You may also use ligase chain reaction or NASPA
6 or you can make use of auto replicated DNAs or RNAs in
7 order to generate amplification product, which can
8 then be assayed by different methods.

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

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

20 Actually as others, we can detect only a
21 few particles in the case of HIV. We believe that in
22 some cases we can detect even less than one particle.

23 Now this is one of the theories taken from
24 the Joerg Koenig paper in 1996, where we demonstrated
25 that the measles vaccines, the mumps vaccines, the

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1 yellow fever vaccines, and the MMR vaccines all
2 contain activity which is about three orders of
3 magnitude higher than the background here on other
4 vaccines, and were negative.

5 Now in order to identify the viruses
6 behind these activities, we along with the PERT assay,
7 developed the method for the identification of unknown
8 retroviruses. It is based on three properties of old
9 retroviruses, namely, that they all are
10 polyadenylated, that R sequences are repeated at both
11 ends, and that cDNA synthesis has started here at the
12 primer, binding site, and that for primers, tRNAase
13 are used and the use of such tRNAase is actually very
14 much restricted among the various retroviruses. For
15 example, is just four PRNA primer equivalence. You
16 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

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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

11 the three prime end. If it is, you can then amplify
12 the entire genome with a little bit of luck by long
13 PCR.
14 So this is what we use to identify this
15 EIV-O sequence. We have also done some other work.

16 For example, we investigated the NIH 323 cell line.
17 This was negative by convention RT tests, but positive
18 by PERT assay. We had a nice band in sucrose, and
19 then radiant. Using this procedure which we call

20 parar, we identified 23 different products, 15 of
21 these were actually retroviral sequences from four
22 different groups. Three of them were unknown

23 sequences, at least at that time. So far we have not
24 further characterized these sequences, but this is
25 still awaiting.

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1 Now staying with retroviruses, as Dr.
2 Coffin pointed out yesterday, sometimes if you have a
3 cell line here, you are dealing with melanoma cell
4 lines which were found to be highly percipated by PERT

5 assay. We analyzed what was in there. It turns out
6 to be endogenous murine leukemia virus, and later we
7 were told that these cell lines have actually been
8 passaged in mice.

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

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

23 Next, please. This is another example of
24 a primary culture where we have a very short peak at

25 the higher density. This might be for particles,

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1 could be a different retrovirus, a different virus, or

2 just a subcellular particles containing some cell or

3 enzymes.

4 Now you will say that this test of course

5 detects only retroviruses that are released. We are

6 also worried about retroviruses that are inside the

7 cells, so stimulation may be necessary. Actually I

8 think one important question is or one possibility is

9 that actually the vaccine virus we would like to

10 produce in such a cell might activate latent

11 proviruses. So I think it is important that we

12 actually do not just test the virus production systems

13 while uninfected, but also when this seed virus has

14 been added, and then we harvest the virus.

15 Now in some cases, as in the measles virus

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

17 specificity. But in other cases, it might be more

18 difficult as indicated in this example, where we

19 tested a vaccine, experimental vaccinia, recombinant
20 vaccinia virus vaccine against melanoma. This was
21 found highly positive by PERT. It had actually been
22 produced by just the lysing, the infected cells by
23 ultrasonication.

24 What we now find is here in black, is the
25 vaccinia virus DNA two peaks. We have here a major

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1 peak of RT activity which does not coincide with the
2 vaccinia virus peaks, and also is not characteristic
3 of retroviruses. So I think in this case, we can rule
4 out the presence of a retrovirus.

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

13 enhancer regions.

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

22 The number of inducers have been described
23 in the past. The most important ones are listed here
24 at the top, allogenated pyrimidins, the azacytidine,
25 which only both of them working only in infected

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1 cells. I will not mention the others because of the
2 lack of time.

3 Now it depends a lot on the virus whether
4 azacytidine or the deoxy pyrimidine is preferable.
5 For example, in experiment in cell line where two
6 types of different retroviruses are produced, several

7 type A particles here. The azacytidine is certainly
8 better. But in C-type particles, these cells produced
9 IdUdr. Yes, the IdUdr is better. So you might have
10 to use a combination of these two drugs.

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

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

23 I also think that at the end, the only
24 important thing actually when dealing with
25 adventitious agents, not just with DNA, which might be

1 infectious, is that the vaccine is free of these
2 contaminant viruses and for retroviruses I believe
3 that this can be verified by the PERV assay. Thank
4 you.

5 (Applause.)

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

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

16 What I think is very important to add to
17 this would be one more level to your last slide. That
18 is an infectivity step. As in the example we saw
19 before when one perhaps collects a panel of cells or
20 cell lines which are pert negative, and there seems to

21 be reasonable numbers of those, and then test the
22 vaccine, the induced stuff and everything else by
23 infectivity and induction of pert activity on those
24 cells.

25 I think that would be a much more useful

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1 and reliable test for the presence of viruses that
2 might be problematic than simply looking at the pert
3 activity in preparations with cell soups.

4 DR. SCHUEPBACH: Yes, I agree with you.

5 I actually thought that was included in those

6 conventional methods which I have listed for the known
7 viruses. Of course you should also do some studies
8 for retro viruses.

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

13 DR. SCHUEPBACH: Yes. These are very
14 recent results. We are in the process of doing that.

15 DR. KRAUSE: Phil Krause, FDA. One of the
16 issues in testing vaccine products is obviously what
17 tests are available and have been validated and that
18 we understand the sensitivity of. So I guess in the
19 context of thinking of highly conserved sequences to
20 which we might develop primers that could detect a
21 broad array of viruses, including some unknown related
22 viruses, what can you say about the current state of
23 the art? How good is that? How well has that been
24 validated? Is that something which if we decided
25 tomorrow we wanted to apply that to new vaccines

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1 producing neoplastic cells, we could simply say “let’s
2 do it” or is more work required?

3 DR. SCHUEPBACH: I’m actually not very
4 familiar with other viruses than retro viruses. But
5 I think these things, however they exist, should
6 clearly be developed.

7 CHAIRPERSON RABINOVICH: Thank you. We
8 will go onto our next speaker, Dr. Jens Mayer, from

9 the University of Pennsylvania. The status of HERV in
10 human cells.

11 DR. MAYER: Okay. My talk will deal with

12 -- can I have the first slide, please? Okay. My talk

13 will tell you something, I hope, about the status of

14 these human endogenous retrovirus regarding the coding

15 capacity and the expressions. Just again, it was

16 mentioned before already what is actually an

17 endogenous retro virus. HERV is created by the germs

18 of infection of an exogenous retro virus. This leads

19 to radical inheritance of this newly created virus

20 following generations. In the course of the

21 evolution, it will be also inherited to newly arising

22 species.

23 The human genome, like all mammal genomes,

24 and also some invertebrates, invertebrate genomes

25 where it has been shown, contains several families of

1 elements and so on. It has been estimated that about

2 one percent of the human genome of such retro origin.
3 These elements antiquated already several million
4 years ago through the genomes of human predecessor
5 species. Some present for at least 30 million years.

6 Some have been shown to be present for at least 40
7 million years. We have several indications of
8 different various families. So they were independent

9 of several exogenous retro viruses. Some of these
10 elements that are now present in the human genome
11 existed. Single copy, and some have copies, copy
12 numbers up to 1,000, per haploid genome.

13 But as I said, most of these sequences
14 were already present for a long time. Therefore were
15 targets for mutations. Most of these families then

16 became coding deficient or they do no longer encode
17 for retro R proteins. However, even if they are
18 coding deficient, many of these families are still
19 transcribed in several human tissues. Some have been

20 discolored just by virtue of their expression.

21 It also seems that the expression of these
22 sequences is regulated in certain tissues and tumors,

23 so we heard that there might be an deregulation of
24 families. It seems possible that that deregulation
25 mechanism is not present in certain tumor tissues.

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

9 I said that most retro viruses are coding,
10 HERVs are coding deficient. However, there are some
11 good described examples, especially some new examples
12 of coding in tact HERV sequences. At least there are
13 some in tact genes. We have already known for a long
14 time the so-called ERV-3 sequence that belongs to the
15 R-family. This, we agreed, pro-virus, or pro-virus

16 sequence encodes, and 1.9 KBN open reading frame.
17 That open reading frame is highly regulated to the
18 transformation of trophoblasts into sensitio-
19 trophoblasts in the placenta. So we have here clearly
20 an up-regulation during a developmental stage.
21 We have for instance, you have H-family
22 and we have about 1,000 copies of that H-family.
23 Among them are 100 copies that are still in tact
24 regarding the pro-virus structures. They have an LTR
25 gag pol env, LTR structure remaining 900 lack N gene.

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1 There has also been reported that this HERV-H families
2 are expressed in various cell lines. We see the
3 highest expression for these elements has been
4 reported in cell lines that are derived from germ cell
5 tumors, and germ cell tumors I guess you will hear
6 some more about germ cell tumors later on.
7 Just this year, Lindeskog, Mark Lindeskog
8 reported the isolation of an intact HERV-H env gene.
9 So it is now clear that there is within the human

10 genome one intact HERV-H evn gene. It's not know so
11 far whether there are any among these many sequences,
12 whether there are any intact gag of pol sequences.

13 I would like to mention the new discovered
14 HERV-W family that has originally been reported, has
15 been isolated from retro virus by particles from

16 multiple sclerosis patients. It has also been
17 reported that these HERV-W sequences are up-regulated
18 in the placenta. Joni Blanc also reported this year
19 the isolation of an intact HERV-W in the genes. It is
20 also not known whether there are intact gag pol genes.

21 I would like to in the second part of my
22 talk, report about results for our family of clearly

23 outlines from our other HERV families in the coding
24 capacity. This is the so-called HERV-K HML-2 family.
25 This is quite complicated.

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1 The human genome contains several families

2 that use lycine primer binding site or TRNA for primer
3 binding. They were named human MMTV-like sequences,

4 one through six. The family that we are talking about
5 is reported in more detail by Ono and co-workers and
6 the original sequence was the so-called HERV-K 10
7 sequence, which is by the new nomenclature is the
8 HERV-K HML-2 sequence.

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

14 In the past, there have been reports of
15 isolation of the isolations of intact HERV HML-2
16 sequences. So there were reports about intact gag
17 sequence and intact protease sequence has been
18 reported, that is able to process that HERV-K gag
19 protein, intact pol sequences with RT activity, with
20 endonuclease activity, and have been reported and also
21 intact mRNA has been reported from the group from
22 Johannes Loewer. And also what we heard yesterday
23 evening, there is also an additional splicing product

24 from the N gene, the so-called C-ORF that still has a
25 rav-like function.

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

18 results, we also know that the precursors of these
19 tumors, the so-called carcinoma in situ, also
20 expresses already on the RNA level these HERV-K HML-2
21 sequences.

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

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

10 We were able to show that there are at
11 least, still at least eight intact gag genes within

12 the human genome, and at least three intact env genes.

13 We did not publish that. There are also several

14 intact pol genes within the human genome.

15 I just want to show you how we got these

16 numbers. This is the protein truncation test that has

17 been described by Roest and coworkers in 1993. So it

18 was originally developed for the detection of APC gene

19 carriers that carry it, the APC gene. So the APC

20 lesion is characterized by trends or not completely

21 translated APC proteins. It is almost like the 3

22 prime terminus.

23 So we have three possibilities. One is

24 that both are intact, both genes are intact. The

25 carrier will carry one defective APC gene. The

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1 defective person would carry the two defective genes.

2 The principle of the test is that the coding sequence

3 is PCI amplified, where the protochomo contains the T-

4 7 promoter and the translation initiation sequence.

5 So if this PCI product is then in vitro transcribed

6 and translated and impregnated and radiolabeled amino
7 acid, electrophoresed, and then auto radiographed, you
8 will see according to the status of these donors that
9 you will have only full-length proteins, the carryover
10 also show an additional shortened protein and
11 defective people will only produce defective proteins.

12 We in principle used the same test because
13 we in principle have the same situation. We have some
14 defective gag genes within the genome. There must be
15 at least one gag or env gene because we have the
16 antibodies. So we put—in principle used the same
17 test.

18 What we did was we are looking for the
19 presence of full-length gag genes or env genes on the
20 human chromosomes and then tested the PCI product we
21 got from the chromosomes for their coding capacity.
22 This is the result for the gag coding capacity. So
23 gag protein would result in a protein of about 73
24 kilodaltons. So these are controls that give the
25 respective proteins.

1 You see that there are several human
2 chromosomes that contain or produce a full-length
3 protein. There are eight human chromosomes that
4 contain at least one gag gene that contains four full-
5 length proteins.

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

12 We did the same for the HERV-K env genes.
13 We see here that three chromosomes produce a protein
14 of about 76 kilodaltons. These are the chromosomes 7,
15 19 in here on the chromosome.

16 What we also see in the gag experiments is
17 that there are additional env genes that are only on
18 the almost intact. We have here a protein that is
19 about four kilodaltons more. So this actually could

20 also be considered as an intact reading frame.

21 So we have several human chromosomes that
22 still contain gag and env genes. We have three
23 chromosomes that contain both intact gag and env
24 genes, the chromosome 7, 19, and the Y chromosome. We
25 were interested whether these chromosomes or the

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1 intact genes on these chromosomes are derived or
2 located within one provirus or within several or
3 different positions within the particular chromosome.

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

10 What we finally found out, that we
11 isolated the so far least defective human endonuclease
12 on chromosome 7. We were able to characterize the
13 proviral sequence within one cosmid clone that still

14 has intact LTRs. So they regulate to the elements.
15 They are able to transcribe, as you will see. We have
16 an intact gag gene. We have an intact protease gene
17 that protease is able to cut itself from a gag
18 protease, polymer precursor protein, and is
19 furthermore able to process encoded gag proteins. So
20 it's typical retro-ized protease.
21 We know just from sequence comparison, one
22 can deduce that the endonuclease within the polymer
23 genes also acted just by sequence comparison, no
24 significant changes compared to recently described
25 active K in the nuclease. We have an intact env gene.

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1 This intact env gene sequence has already been
2 described by Johannes Loewer's group as an MRA, which
3 also shows that this sequence is actively transcribed.
4 So this is actually an expressed provirus.
5 We have spliced on the inceptors sides the
6 corresponding position that would allow to splice an
7 M on A, and what we heard yesterday also, to splice an

8 additional soft M RNA.

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

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

21 We have no infectivity so far shown for
22 any of these HERV sequences. We do not really know
23 why. There are several reasons that can be mentioned
24 for the HML-2 family. It has to be reported that the
25 env protein cannot be cleaved into the auto membrane

1 transmembrane domains. It is conceivable that they

2 are defective genomes that are packaged into these
3 particles, so only if they would be able to get a new
4 cell, they would only deliver defective genomes.

5 It is also not clear whether the
6 receptors, that they were once used by that, retro
7 families are still present and would still be used.

8 So what you should take home I guess is
9 that human endogenous are expressed in several tissues
10 tumor types that are highly up-regulated in certain
11 tumor types. Several HERV families are still able to
12 encode proteins, and among them, the HML-2 family that
13 still encodes all essential proteins. We have almost
14 intact HML-2 provirus within the human genome. Thank
15 you.

16 (Applause.)

17 CHAIRPERSON RABINOVICH: I think we will
18 hold questions at this point. We are going to take a
19 10-minute break now. We are going to come back and
20 finish with the last two speakers.

21 I need to figure out how to catch up time,

22 and yet leave the time for the panel discussions. I
23 ask you to do two things. Check-out time from the
24 hotel is 12:00. You should know that. They have
25 already called in a bunch of the taxis so that if you

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1 need taxi arrangement, please let them know so they
2 can do that for you. Ten minutes we will start again.

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

6 CHAIRPERSON RABINOVICH: Is Dr. Broker
7 here? Great.

8 If you could take a seat please. The next
9 speaker is Dr. Thomas Broker from the University of
10 Alabama at Birmingham speaking on viral latency-
11 papilloma virus model.

12 DR. BROKER: Thank you very much. I would
13 like to deal with two subjects under this topic. The
14 first is a study of the prevalence of HPV in the
15 general population, and then following on Dr. Mayer's

16 pattern that you just heard, a study of some
17 endogenous sequences in papilloma virus transformed
18 cell lines with some surprising results.
19 We have done some inside 2 hybridization
20 studies of the expression of human papilloma viruses
21 in biopsies from women with HIV/AIDS who were
22 moderately immuno deficient. This is one example, but
23 fairly typical.
24 What you are seeing is a full thickness of
25 across the cervix. The various probes that we used

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1 reveal the expression of one of the major early
2 transcripts of papilloma virus, the E4, E5. You are
3 seeing it here in bright field illumination and dark
4 field, matched pairs, basal layers right there.
5 As I indicated yesterday evening,
6 papilloma transcription is differentiation dependent
7 and occurs typically in the upper half of the skin.
8 E6, E7 messages, the delayed early oncogenes are hard

9 to see in bright field, but fairly easy to see in dark
10 field. You can see they follow a comparable
11 distribution.

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

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

24 We decided to investigate the prevalence
25 of HPB in the population by focusing on immuno-

1 deficient groups. The three that we have chosen so
2 far are: women who are in enstay renal failure and in

3 need of a kidney, and most clearly ill; those then who
4 get a kidney and are pharmacologically immuno-
5 suppressed beyond their underlying illness; and those
6 with AIDS.

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

19 So basically we start with the nested PCR
20 approach, outer primers and inner primers. The
21 starting material is cervico vaginal lavage, which
22 harvests cells from throughout the lower genital tract

23 of the women. We amplify and then we put it through
24 several different assays. Initially, agarose gel
25 electrophoresis to look for a 278-base amplicon.

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

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

14 The study is longitudinal, and has been
15 going on for three-and-a-half years now. Many of the
16 members of the cohort have been sampled two up until

17 seven different times at six to 12 month intervals.
18 So that's our biggest cohort.
19 These are the renal transplant cohort. We
20 have statistically significant numbers. I would like
21 to point out that in instage renal failure, but no
22 pharmacologic suppression, about 59 percent of those
23 women have detectible HPV. Again, quite a few, a high
24 percentage have multiple infections. This carries
25 over to that portion of this group who go onto actual

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1 transplantation.

2 Some of the remarkable outcomes of the
3 care with which we undertook the genotyping is the
4 following. In the pre-transplant population, the
5 prevalent types are those that are commonly seen in
6 the general population as causing disease, namely HPV-
7 6, 11, and 16. Those types persist in those women who
8 were pharmacologically immuno-suppressed.

9 We see a scattering of other types, but
10 the common types from prior studies are those that

11 predominate in the renal transplant cohorts.

12 In contrast, those women who are in

13 various stages of immuno-deficiency as a result of

14 AIDS, do not show the same genotype profiles. The

15 only member in common is in fact most common of all

16 genital HPVs, HPV-6. What we see instead are niche

17 homologs of the common types. For example, HPV-45, as

18 you are going to see, is a close relative of HPV-18,

19 which is often cited as a common virus. But we don't

20 see that in the AIDS cohort.

21 HPV-52 is our most common virus. It is a

22 close homolog of HPV-16, which we don't see amplifying

23 in this cohort. Most notably are the ones that I

24 indicated by stars, which are a very rare detection

25 within the general population, but in fact are most

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1 common viruses in the AIDS cohorts.

2 In particular, we have identified 13 new

3 HPV types based on less than 75 percent sequence

4 homology to each other or to any other known papilloma

5 virus. They are all members of what has been
6 designated group A-3, which appear to be an AIDS-
7 defining subset of HPVs.

8 These can be at least considered in the
9 context of phylogenetic trees based on sequence
10 alignments in the L1 region. So, for example, HPV-16,
11 the main cause of cervical cancer in the world, is
12 seen in the renal cohort, but a very close relative,
13 52, is seen in AIDS.

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

18 HPV-70 is one of our most common types, as
19 well as 45. They are in the HPV-18 family, but
20 represent new members of this niche. The group I just
21 mentioned, A-3, that is so commonly seen in AIDS,
22 include our members jyn 2, 3, 4, all the way up to 13,
23 MM8 and 61, 72, and 83. That cluster seems to be an
24 AIDS defining group. The other ones that we have seen

25 abundantly are 51 and 53 in this arm.

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

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

16 It is known that these patients all have
17 particular cell-mediated immune deficiencies. Again,
18 suggesting that particular arms of the immune system

19 are responsible for either containing or failing to
20 contain different subgroups of the papilloma viruses.
21 As we look at these women over a period of
22 time through these six month or so samples, what we
23 also find, and other labs have exactly the same
24 results, is every time we sample, you may or may not
25 see the type you saw before. It may switch. For

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1 instance, we have this patient who had 6 plus 16, and
2 then 11 plus one that was minor and we couldn't tell,
3 then jyn 2, and then type 40, and then we had a type
4 53, but the others disappeared.

5 Everyone's experience in the field has
6 been that the viruses rise above a detectability
7 threshold, stay there for a while, days or weeks or
8 months, and then fall below detectability, only to be
9 replaced by a different HPV type. These are not new
10 infections. They are basically cryptic or latent
11 persistent infections that fluctuate in their levels
12 of replication and detectability. Pretty much anybody

13 is showing that flexibility.

14 What I want to state at this moment before

15 showing the correlation with disease may sound

16 controversial, but I will stick by it. We have found

17 a brand new HPV type for every 10 people that we have

18 looked at. Philodelius and Ethel Michelle Diveres and

19 zur Hausen and Shamen in European study of tutanius

20 papilloma viruses have found a new papilloma virus for

21 just about every other person they have looked at when

22 they use the combination of nested PCR and DNA

23 sequencing.

24 Robbie Burke's group, Jill Polefski's

25 group, have very comparable experiences looking at

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1 anal papillomas or female genital tract.

2 It is my contention right now that instead

3 of 80 HPV genotypes or 150 that have been officially

4 named, that there probably are millions of variants,

5 virtually a continuum. We feel that basically

6 everybody has their own personal micro flora, that

7 these are passively acquired or vertically acquired,
8 not necessarily sexually, but certainly possibly
9 sexually, and that they simply are part of the human
10 condition as are microflora, just as we have
11 microflora composed of bacteria and many other
12 viruses, and that they basically are utterly
13 ubiquitous. I will come back to that point in a
14 moment.

15 We did try to correlate the various other
16 medical parameters in these cohorts, especially the
17 AIDS cohort, with CD4 count, HIV virus load, other
18 infecting known STDs like herpes, chlamydia,
19 trichomonas, so forth. The one correlate that held up
20 and not surprisingly at all, was that the degree of
21 pap smear abnormality from normal, abnormal cells of
22 unknown significance, low grade dysplasias or high
23 grade dysplasias, is with CD4 count.

24 The medians, these are all the people who
25 had multiple infections, a high risk virus type, a low

1 risk, no virus at all, and had either normal or these
2 various abnormal pap smears. These bars here are the
3 median CD4 count in each of these groups.
4 The one place where we saw active disease,
5 low and high grade dysplasia, these by median, is when
6 people fell below the CD4 count of 200 cells per cubic
7 millimeter.
8 In summary of that data, we found that
9 it's very very possible to have negative pap smears,
10 but definitely have HPV infections. We feel these are
11 people who have not yet reactivated long enough to
12 have resulted in cytologic change as a result of
13 infection. We have on the other hand, the people with
14 overt disease by biopsy or by cytology, and the higher
15 the grade lesion, the more likely it is to see either
16 single infection or especially multiple virus types
17 present within that patient at that time.
18 So the more that we can detect the virus,
19 that is, the more it has replicated or amplified
20 throughout the population, the more cells that are

21 shedding the virus in effect, the more likely we see
22 disease.

23 So to summarize this part of the talk, I
24 feel that they are virtually ubiquitous. they are
25 typically sub-clinical, persist in or latent

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1 infections. There are staggeringly large number of
2 genotypes if we take the care to look. I might say
3 that the reason these are typically not found is that
4 people use generic cross-hybridizing probes or have
5 cut off their probe sets. If you're not probing for
6 something, you are not going to see it.

7 Most of the viruses in this number 60, 70,
8 80 and above, are not even present within the
9 commercial probe sets. So if you aren't probing, you
10 are not going to see them, and you are going to get
11 lower numbers.

12 They can be found throughout the genital
13 tract in 60 to 75 percent of the people that we have
14 looked at who are admittedly good yielders, because

15 they are immuno-compromised, but I think this simply
16 represents the general infection in the population.
17 They can be found in oral and esophageal
18 mucosa. Ubiquitous types persist in hair follicles.
19 There's a wonderful study from Amsterdam by Tershaegt
20 and Ingebor Boxman. She plucked hair follicles, both
21 eyebrow hairs and pubic hair, and 60 to 70 percent of
22 all people harbored EV viruses or other rare virus
23 types in their hair follicles. No disease, it's just
24 part of the human condition.
25 I believe they are vertically transmitted

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1 perinatally, mother to baby. Some of them are clearly
2 pre-natal infections. As we know, there's long-term
3 maintenance that requires viral replication in concert
4 with host replication in the cell cycle.
5 So what I would like to do now is tell you
6 a little bit about a very unexpected observation we
7 made in Hela cells. This goes back to last night's
8 talk regarding the structure of the replication

9 complex of HPVs.

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

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

20 In other words, HPV infection upregulates cyclin E.

21 So we asked whether the induction of
22 cyclin E is essential for the reactivation of
23 unscheduled cellular DNA synthesis in the upper
24 stratum of squamous epithelium that differentiated
25 keratinocyte. I'll just summarize that data.

1 I got you to the point last night where

2 the E1 dihexamer, the double helicase held together by
3 the HSP-70 cochaperone protein, is there.

4 The next thing that loads in the study we
5 did with Theresa Wong at Stanford, is the recruitment
6 of the cellular DNA polymerase, and showed direct
7 interactions between the helicase and the catalytic
8 sub-unit of pol alpha, P-180, as well as its P-70 sub-
9 unit. This was the first indication of what P-70 does
10 in the four sub-unit complex of pol alpha, which
11 includes two primary sub-units. The answer is, it
12 brings the polymerase to the ora itself.

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

22 Secondly, E1 is phosphorylated. These two

23 subunits of preliminary salpha that bind directly to
24 E1 are phosphorylated. When all four of those have
25 been successfully modified, the kinase phosphorylates

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1 cyclin E itself, which is displaced and degraded by
2 ubiquitination. That enables the pre-initiation
3 complex to convert to the elongation complex.

4 In studies with Wade Harper and Jien-Ling
5 Ma at Baylor, two things were done. The first is
6 together we found that there's a cyclin binding motif
7 that the amino terminal have at the E1 protein, which
8 in fact is shared with a number of other things that
9 bind the cyclin E. That motif involves an RXL. That
10 is, an arginine something leucine motif right there.

11 In addition, their candidate
12 phosphorylation sites, the series of serine, serine,
13 serine, and threonine, mutation of any of these, the
14 motif or any of the target phosphorylation sites,
15 diminishes the capacity of cyclin E to convert the

16 pre-initiation complex to an initiation complex. So
17 the functional requirement for phosphorylation has
18 been verified. But keep in mind this location. We'll
19 come back to it in a second.

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

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1 we monitored cyclin E expression, over expression in
2 the tissue. Here's bromo deoxy uridine incorporation
3 or PCNA upregulation. These are the match.

4 What we found is the cells that had high
5 cyclin E could not replicate. In fact, they are
6 mutually exclusive with those capable of supporting
7 DNA synthesis. Conversely, PCNA, which is upregulated
8 by papilloma 7 and cyclin E do co-localize. But we
9 see a number of cells where PCNA is present and there

10 is no cyclin E. So we have a reciprocal pattern to
11 what we expected.

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

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

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

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1 So ironically, the cells that have high
2 cyclin E also have high P-21 and do not support
3 replication. This was really perplexing, except we

4 did know this inhibited that. But we assumed cyclin
5 E was in the licensing factor for engaging in
6 replication.

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

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

21 Now we put this all together by asking how
22 does this play into the establishment of immortalized
23 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 cHa 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

18 expressed a full length E-1 transcript, but in all
19 cases, the transcript had an either frame shift or a
20 stop code on partway through the gene or miss sensed
21 mutations in this vicinity, so that in tact E-1 could
22 not be made. But in all cases, it made the RXL
23 portion that interacts with cyclin E.

24 So we added a little cyclin E back to hela
25 cell extracts and immediately restored full

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1 complementability to those extracts, establishing that
2 the missing thing in hela cells was cyclin E.

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

11 So what these natural experiments did, is

12 figured out a way to sequester part, but not all, of
13 the cyclin E by putting in retaining fragments of E-1,
14 capable of mopping up that one product that's
15 upregulated that you don't want to have. That is,
16 cyclin E.

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

22 Together we believe that to establish
23 these cell lines, whether you make them in the lab or
24 whether nature has made them for you through cancer,
25 you need to diminish the amount of cyclin E to achieve

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1 an equilibrium where you have enough cyclin E to
2 support cellular replication, but not too much so that
3 it's an unsuccessful high level. Thank you very much.

4 (Applause.)

5 Oh, I did want to acknowledge—could I

6 have the slides for one second—a very, very large
7 number of collaborators. I'll leave them up here.
8 But we're very grateful for our own students and
9 collaborators at UAB, Jeff Engler, Doug Seer, Sean Van
10 Tine, Kim Towns. At Baylor, Wade Harper's lab, UNC,
11 Jack Griffith's lab, who did the things with the HSP-
12 40, and at Stanford, Theresa Wong, and our
13 collaborators at the Free University, who did a lot of
14 work on some transcriptional control that we
15 collaborated on for several years. Thanks.

16 CHAIRPERSON RABINOVICH: Thank you. Are
17 there any questions?

18 DR. FRIED: How could you be so sure that
19 with the evolution of the HPVs, are not due to new
20 infections, but to pre-existing sequences to cover?

21 DR. BROKER: I think one is the repetity -
22 -

23 DR. FRIED: Mike Fried.

24 DR. BROKER: With which these viruses are
25 appearing, especially in women who at least declare

1 they are not engaged in much, if any, sex. A number
2 of the epidemiologic studies have traced either the
3 frequency of recent sexual activity. Everyone who has
4 looked feels its an emergence of pre-existing
5 subclinical infections. There is some evidence in new
6 infection, but an awful lot of it simply appears.

7 Women in that stage renal failure, for
8 example, who are 38 years old and married basically,
9 are not suddenly acquiring new infections.

10 CHAIRPERSON RABINOVICH: Back microphone,
11 please. Why don't you come up to the front.

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

13 Very interesting talk.

14 I have a couple of questions. One is, how
15 can you be sure that what you are sampling by PCR is
16 really an infective virus, it's not just a transient
17 presence with a virus due to the fact that you can't
18 control sexual behavior, and perhaps the woman has
19 just been exposed to a virus?

20 DR. BROKER: Well, I think one way is that
21 in a very high percentage of the people, there are
22 clear dysplasia, low and high grade. All the women
23 who have any degree of dysplasia are also biopsied,
24 and the inside 2 hybridization, as you can see, is
25 showing clear effects in the tissue.

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

5 DR. RUSSO: I may have missed the data.
6 Did you show the types that are associated with high
7 grade lesions, the HPV types? I didn't see on the
8 table.

9 DR. BROKER: Yes. Well basically, in this
10 immense spectrum of what's now 37 different viruses
11 that we found, those that are most typically
12 associated with low and high grade dysplasia, the
13 actual diseases, are the higher risk types.

14 DR. RUSSO: So you are not suggesting that
15 if you want to prevent cervical cancer, we should
16 focus on different types of the one already
17 identified?

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

22 The one thing that I think is going to --
23 and I commented a day or two ago that in the U.S.
24 alone today, there are over 250 to 300,000 people
25 immuno-suppressed just due to organ transplants,

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1 steroid use, or bone marrow transplants or AIDS. So
2 there is an immense reservoir of particularly high
3 risk patients.

4 Nonetheless, most of the diseases are
5 still being caused by a handful of viruses like 16,
6 18, 52. So I think, at least the ones we have to
7 worry about today, are still manageable in number.

8 AUDIENCE MEMBER: I would like to ask a
9 little bit about cell substrates. Considering that
10 hela has multiple HPV integrants, I guess, are any of
11 those infectious?

12 DR. BROKER: No.

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

15 DR. BROKER: No.

16 AUDIENCE MEMBER: What are they missing?

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

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

23 DR. BROKER: They do contain the E-6 and
24 E-7 genes. Expression of those genes, as shown by
25 studies primarily in zur Hausen's lab, must be

1 maintained or you no longer can cycle hela cells.

2 That is antisense to E-6 and E-7 in hela makes them
3 not cycle any more. So the driving force of hela is
4 the overt expression of E-7.

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

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

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

14 (Laughter.)

15 DR. MURPHY: This is Murphy from NIAID.
16 That is, do you see any reason why, you know, having
17 an intimate knowledge of hela cells and human
18 papilloma viruses, that hela cells should not be used
19 as a substrate for making live attenuated virus
20 vaccines?

21 DR. BROKER: I don't know of any evidence

22 of these genes being transduced out or in any way
23 posing a risk. I was going to save it for the panel,
24 but it occurred to me last night, I had challenged one
25 of last night's speakers about the use of psorilins as

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1 a cross-linking agent. It gives me great concern that
2 it is a known carcinogen. However, Brian and I were
3 talking. Brian van Tine and I were talking last
4 night, and he reminded me that there are biotinylated
5 psorilins.

6 So for all the debates regarding how to
7 remove contaminating DNA, one strategy in principle is
8 throw in a biotinylated psorilin, cross link it, and
9 pass the whole thing over avidin magnetic beads or
10 batch subtraction of the DNA.

11 So in fact, that strategy may actually
12 help you deplete adventitious contaminants very, very
13 readily. So it is an alternative at least.

14 AUDIENCE MEMBER: Can I just ask you about
15 hela again? We learned last night that not every HPV,

16 if there's 30 to 50 copies, are not all active, I mean

17 in caski only one was active.

18 DR. BROKER: Yes.

19 AUDIENCE MEMBER: What is the state of

20 hela? Are they --

21 DR. BROKER: Very, very few are active.

22 We and Wade Harper's lab are both sequencing all the

23 transcripts. This actually was done by Elizabeth

24 Schwartz and others in zur Hausen's lab in 1985, and

25 a variety of groups since then in Japan and elsewhere

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1 have looked at the expression loci in copies in hela.

2 There appear to be three or four different

3 transcripts made from different positional integrants,

4 but the majority are silent. A few of them are

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

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

7 but they don't have their normal carboxy terminus.

8 CHAIRPERSON RABINOVICH: Thank you very

9 much.

10 Let's go on to Dr. Cashman. Thank you for
11 being so patient. Transmissible spongiform
12 encephalopathies: vaccine issues.

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

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

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

24 I want to spend a few minutes talking
25 about prions and prion disease. We had a speaker

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1 yesterday who said "and now for something completely
2 different." Well, how does a genomeless infectious

3 agent grab you?

4 Creutzfeldt-Jakob disease is the most

5 common human prion disease that we run into. I do

6 want to spend a few minutes talking about this so that

7 we are all on the same page with regard to public

8 health risks. Creutzfeldt-Jakob disease or CJD is

9 basically a disease you wouldn't want to wish on your

10 worst enemy. It is a completely untreatable uniformly

11 fatal disease resulting in death within six to nine

12 months of presentation. Survival over a year is

13 recorded, but it is not very frequent.

14 The presentation is usually that of a kind

15 of Alzheimers-like syndrome, with problems in memory

16 and intellectual function, but it can also present as

17 a disorder of gait and balance as well. Most people

18 have mild clonus, which is twitching of the muscles,

19 sufficiently forceful to move a joint. Other features

20 of the neuro-degenerative syndrome are reminiscent of

21 other neuro-degenerative diseases like Lou Gehrig's

22 disease and Parkinson's disease. Basically it's like

23 having every neuro-degenerative disease at once,
24 telescoped into an unmercifully short period of
25 decline.

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1 Fortunately, it is rare. Sporadic

2 Creutzfeldt-Jakob disease occurs at about one per
3 million population per year. Also, somewhat
4 fortunately, it's not a disease of children. The
5 average incidence of CJD is in the 60s.

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

16 Of course the transmissible spongiform

17 encephalopathy that even my kids know is bovine
18 spongiform encephalopathy, or so-called mad cow
19 disease. Since the early 1980s, this disease has
20 affected about 200,000 cattle in the U.K. and Republic
21 of Ireland, and a few hundred across continental
22 Europe. About 2 million cattle have been killed in an
23 attempt to stem the epidemic. This culling, as well
24 as change in policies, such as feeding ruminant to
25 ruminant—we turned cattle into neo-cannibals—is

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1 resulting in a rapid decline of new cases, predictions
2 being that the epidemic in cattle may be essentially
3 stamped out in the early part of the new millennium.
4 I won't even say the new century. I'll say the new
5 millennium.

6 Unfortunately, this disease is unique,
7 unlike every other known naturally occurring prion
8 disease. It doesn't seem to obey species barriers, or
9 at least obeys them to a much lesser degree. There is
10 an outbreak of feline spongiform encephalopathy in

11 house cats. There is spongiform encephalopathy in zoo
12 animals, including primates. The primates that we are
13 most concerned about are also vulnerable to this
14 disease.

15 To date, 44 people have developed a new
16 variant of Creutzfeldt-Jakob disease, which is
17 clinically and pathologically distinct from classical
18 CJD. The statisticians predict there will be
19 somewhere between a few hundred and maybe 80,000
20 cases. This does not include the chicken little
21 predictions of the extent of the epidemic.

22 The disease unfortunately seems to strike
23 the young. There have been teenagers involved. It is
24 a relatively slower progression than classical CJD.
25 There are clinical features that are distinctive, but

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1 I won't bore you with them this talk. The pathology
2 is also absolutely distinct, including a preter
3 natural accumulation of PrP Sc, which is this abnormal
4 amyloid protein that's been linked to infectivity.

5 This occurs both in the brain and in peripheral

6 lymphoid tissues.

7 Well, before we leave the clinical stuff

8 about CJD and prion disease, I want to kind of set the

9 stage with a sobering statistic, which is there is

10 iatrogenic transmission of this group of diseases.

11 Considering the penetrants and the young age of

12 vaccinees, this is a scary possibility. This would

13 dwarf every other iatrogenic transmission known to

14 date.

15 In humans, basically a few hundred cases

16 have been attributed to iatrogenic transmission, from

17 hormones extracted from cadaver pituitaries, from dura

18 mater transplantation, which is the tough lining of

19 the brain. But incredibly, the largest iatrogenic

20 transmission known to date, also the first documented,

21 was that in passage with a vaccine, which was a

22 vaccine for looping ill of sheep. Formal and

23 inactivated brain preparations passed sheep scrapie to

24 about 1,000 sheep. So hopefully this will not be a

25 pattern with human vaccines.

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

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

16 Now this normal cellular protein can adopt
17 an alternate confirmation, which is rich in beta sheet
18 structure. When this protein is in this alternate

19 confirmation, it acquires many unique physical
20 chemical properties. It becomes partially protease
21 resistant. It tends to aggregate. It's very poorly
22 soluble. Plus, it then seems to act as a catalyst for
23 recruiting more confirmational copies of itself.
24 Now whether this occurs by a kind of
25 enzymatic confirmase activity or whether this is kind

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1 of a biological crystallization phenomenon is being
2 actively investigated. But it is clear that this
3 abnormal confirmation isoform of the protein, called
4 PrP Sc for scrapie, is capable of recruiting more
5 confirmational copies of itself from the normal
6 cellular isoform.
7 So onto vaccines. There are some concerns
8 about vaccines. I will mention three areas that need
9 to be considered. I will dwell most of the time on
10 cell substrates, which is nice of me considering this
11 is a cell substrate meeting. I will also talk briefly
12 about potential prion infectivity coming over in media

13 supplements for those cells, and in excipients, which
14 are compounds used to stabilize vaccines in their
15 final formulation.

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

23 This may be due to the fact that cell
24 lines have very little PrPC, which is the precursor
25 for PrP Sc. The conversion of the protein from PrPC

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1 to PrP Sc forms occurs at the surface or a post-
2 surface compartment. So in general, cell surface
3 abundance of the protein correlates with infectivity.
4 Most cell lines in my own laboratory, including hela,
5 express no more than one-tenth of the amount of cell
6 surface PrPC that a primary neuron does.

7 It has also been thought that the very act
8 of cell division itself can kind of sterilize a
9 culture because cell division can out pace the
10 relatively slow conversion and processing of PrP Sc.
11 So if you have a couple units of infectivity, they get
12 progressively diluted by having huge numbers of cells
13 that bear no infectivity. Finally, those cells may
14 die, the infected cells.

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

24 Now to make some kind of estimate about
25 the spontaneous development of prion infectivity in a

1 cell culture, especially a vaccine cell culture that
2 may have hundreds of trillions of cells, I am going to
3 back up and try to explore some assumptions about the
4 spontaneous development of prion disease in humans,
5 which is the species for which we have the best
6 numbers.

7 According to the prion hypothesis, an
8 occasional accidental mis-folding of PrPC to PrP Sc is
9 what triggers the recruitment process which proceeds
10 on an exponential basis. Each molecule that's
11 converted converts to more, da, da, da, da, da, da,
12 which happens on a post-translational level. No
13 genome involved. "Look, ma, no genome."

14 But sporadic disease in humans is
15 incredibly rare, one per million people per year.
16 Humans have something on the order of 100 billion
17 neurons. So one can make the kind of interesting
18 calculation that a productive infection arising from
19 a single neuron, you need about 10 to the 17th
20 neurons, 100 million neurons across a million people

21 in order to develop spontaneous scrapie.

22 But of course that's not the only way one

23 could potentially develop spontaneous CJD. Discovered

24 by familial CJD and familial prion diseases, there are

25 mutations within the open reading frame of the prion

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1 protein that apparently predispose to this accidental

2 misfolding, such that somebody with a prion protein

3 mutation that actually results in an amino acid

4 substitution is basically guaranteed of developing the

5 disease over the course of a lifetime if he or she

6 lives long enough.

7 So could this occur in vitro? Could

8 certain cells in vitro acquire a somatic mutation

9 which is then propagated to infect an entire culture,

10 again, on a post-translational level? Well, let's run

11 some numbers on this one.

12 The mutation rate in man is about one per

13 billion basepairs per year. I thank Dr. Kazazian for

14 yesterday for pointing me to this reference. Thank

15 you very much.

16 The prion protein open reading frame is
17 really less than 1,000 basepairs. It's a relatively
18 small protein that's all contained in one exon. This
19 gives rise to a kind of pseudo calculation that a cell
20 can develop a mutant prion protein gene, a cell in
21 vivo, and a human can develop a mutant prion protein
22 gene in about one out of a million cells per year, if
23 you take one out of a billion and multiply it by
24 1,000, that's one out of a million.

25 This, just as an aside, this gives a rise

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1 to a kind of startling calculation that all of us in
2 the audience are generating about 100,000 prion
3 mutants in our brain per year.

4 However, there must be a safety factor
5 here because the rate of prion disease arising from
6 somatic mutation cannot exceed the calculation of
7 prion disease arising from individual neurons that we
8 just went through in the last slide, which is 10 to

9 the 17th neurons per year.

10 So incredibly, somatic mutation is a
11 seriously flawed non-efficient process for producing
12 prion disease. Something on the order of one out of
13 100 billion mutations are productive of infection.
14 This may give us some comfort when we turn to the in
15 vitro scenario.

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

25 Substrate cells, however, are less

1 genomically stable than primary neurons. If one says

2 that there's say 1,000-fold greater rate instead of
3 one out of a billion basepairs, one out of a million
4 basepairs can be mutated per year in a substrate cell,
5 this gives rise to a calculation suggesting that you
6 need 10^{14} substrate cells per year in order
7 to have one productive, i.e. spontaneous infectivity
8 arising in a culture.

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

18 So perhaps it is possible, considering the
19 enormous scale of substrate cell culture, that prion
20 infectivity could arise through somatic mutation in a
21 substrate cell, and could contaminate a vaccine
22 destined for human beings.

23 Well, there are some things to talk about
24 with this model. If this is so, how come we haven't
25 seen any vaccine transmissions yet? One of the

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1 factors is that very few human cell line vaccines have
2 gone into humans, certainly not on a scale of vero
3 cells or primary cells that are used for culture.

4 Another unsettling thing is that if indeed
5 there is somatic mutation in a culture of human
6 substrate cells, would we ever detect it? We are
7 talking about something that would occur in one out of
8 a million cells or even one out of a thousand cells,
9 would ever be able to find by PCR or SSEP or anything
10 you could think of, a mutation at this level.

11 So aside from substrate cells, I did want
12 to touch upon a few potential sources of infectivity.
13 The media coming in contact with substrate cells are
14 potentially carrying prion infectivity. Bovine serum,
15 fetal calf serum, and newborn calf serum is used as a

16 supplement for proteins and growth factors and
17 hormones for most cell lines. Some cell lines are
18 also supported by human serum albumin. I am not aware
19 of a vaccine cell line that's supported in this
20 manner, but many recombinant proteins are supported
21 with human serum albumin.

22 There's also potential prion infectivity
23 in excipients, this last compound that's added to the
24 preparation to keep it stable before use. Many
25 childhood vaccines are stabilized with pig skin

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1 gelatin. Pigs don't seem to be a species which
2 spontaneously develop prion disease or a species that
3 can catch prion disease via the oral route, although
4 deliberate intracranial injection of BSE infectivity
5 can produce a prion disorder.

6 Human serum albumin is also an excipient
7 in measles, mumps, rubella, and rabies vaccines. I
8 would like to spend just a few seconds talking about
9 human plasma proteins before I close, and give my

10 final advice, such as it is.

11 Human serum albumin of course comes from

12 humans. Of course it's a plasma fractionation

13 product. There has been a great deal of work trying

14 to identify potential risks of transmission of CJD

15 from human to human through blood or blood products.

16 Suffice it to say that population studies, case

17 control studies, and cohort studies have proven

18 universally negative. There is no documented

19 incidence of human CJD, classical CJD being passed

20 through blood or blood products.

21 There are of course case reports of people

22 getting a transfusion and developing CJD, but one

23 should not expect that transfusion or administration

24 of a blood product is protective against CJD. The

25 incidence of CJD in the transfused or treated

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1 population is the same as in the non-transfused or

2 treated population.

3 However, we're in more difficult territory

4 with variant CJD. This is, I told you, an unusual
5 agent. It seems to cross species lines with impunity.

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

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

15 The agent itself has odd properties. It
16 is stable across species. It may in fact be
17 specialized or selected. I realize that these terms
18 are not often applied to a protein. I'm borrowing
19 terms from agents that contain a genome. It suggests
20 that this agent may be more virulent, especially with
21 regard to peripheral exposure. In other words, one
22 unit of classical CJD infectivity will not cause
23 disease when injected intramuscularly. One unit of

24 variant CJD infectivity may very well. There is no
25 data.

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1 Another thing that has been noted is the
2 young age of onset of variant CJD. This has been
3 attributed to kids eating hamburgers and all kinds of
4 weird meat products. But it could also be attributed
5 to host factors which would promote infectivity in the
6 young. Since vaccinees are usually young, we have to
7 take this in mind.

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

16 This could be done two ways. The
17 biochemical marker of infectivity is PrP Sc. This

18 technology is in evolution. It appears that capillary
19 electrophoresis, some types of optimized immuno
20 blotting, and even ELISAs are reaching the point where
21 one unit of infectivity will be detectible.

22 Another important way of assay for
23 infectivity is called the bio assay in the field, in
24 which selected samples are injected into a species
25 which is capable of supporting that infectivity. That

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1 would be non-human primates, and again, a technology
2 in evolution, transgenics engineered to express human
3 and perhaps bovine PrP.

4 There is also the possibility of trying to
5 prophylax cultures, substrate cultures with chemical
6 agents. This is also in evolution, but the classic
7 molecule in this regard is congo red, which not only
8 seems to bind to PrP Sc, but seems to dissolve
9 infectivity in vitro. New discoveries out of Byron
10 Kohe's lab that tetrapyrrole, including porphyrins and
11 phthalocyanines, can block infectivity. Perhaps some

12 of these compounds can be utilized at appropriate
13 concentrations to use as a kind of antibiotic for
14 substrate cultures.

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

23 So I would like to summarize by saying
24 that it is possible, although not favorable, for
25 substrate cells to be infected with prions. It is

1 possible, considering the huge bulk of cells that are
2 cultured, 300 million a year for one vaccine, that
3 prion infectivity could potentially emerge by
4 misfolding and/or somatic mutation in vitro.

5 I will note that prion components and

6 excipients may contain prion infectivity. Although
7 this is an old story with regard to classical CJD, we
8 don't have the information for the BSC variant CJD
9 agent. We should worry, at least for the time being.

10 The remedies for this are selective
11 sourcing, avoid animals and people that could
12 potentially be brewing prion infection, biological
13 manipulation in vitro, including anti-prion agents,
14 and maybe ablating the prion gene, and then validate,
15 validate, validate. Prion infectivity should be added
16 to the list of infectivities that are excluded in
17 vaccine lots.

18 I thank you for your attention, and I
19 would be glad to answer any questions.

20 (Applause.)

21 DR. KRAUSE: Phil Krause, FDA. In keeping
22 with the idea that one presumably wants to find cell
23 substrates which carry the least risk, I guess if one
24 presumes that tumor cells have a greater risk of
25 genomic instability than non-tumor cells, are you then

1 implying that there's a greater sort of spontaneous
2 mutation than prion risk from tumor cells than for
3 instance primary or diploid cells?

4 DR. CASHMAN: That's a good question. I
5 guess this is basically not quantifiable. If one
6 takes a rock solid cell that enjoys all kinds of DNA
7 repair mechanisms then yes, that is less likely to
8 give rise to the mutation in the prion protein gene.

9 One area which should be investigated, I'm
10 realizing from this meeting, is to take some cell
11 lines and look at 1,000 clones a piece and see if any
12 of the prion copies have acquired mutations. So this
13 would be a piece of data that we could use to actually
14 discuss this issue. Right now, I don't have any.

15 AUDIENCE MEMBER: Along the same lines, I
16 guess the implication would be that if somebody wanted
17 to produce a vaccine in cells that are derived from
18 neurons, given the fact that they are making a lot
19 more of this, you have the potential for greater RNA

20 polymerase mutation rates, and perhaps also greater

21 risks?

22 DR. CASHMAN: Well, neurons are the best

23 factoring for making infectivity, both in vivo and in

24 vitro. Part of that is due to high levels prion

25 protein. Part of it is due to factors that haven't

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1 been identified as of yet, prion receptor, trafficking

2 of prion protein. So I guess one would want to stay

3 away from neurons unless they came from an animal or

4 human that had the prion gene locked out or not

5 transcribed or translated.

6 CHAIRPERSON RABINOVICH: Dr. Egan?

7 DR. EGAN: As Dr. Minor mentioned before,

8 Japanese encephalitis virus is made in mouse brains.

9 Can the PrP SC of a mouse catalyze the conversion of

10 human PrPC to PrP Sc?

11 DR. CASHMAN: That is an extremely good

12 question. I neglected to mention in my talk, my

13 oversight, that there is a very prominent species

14 barrier between most prion agents, something like one
15 to a thousand, one to a million, even higher for
16 generating infectivity, especially between widely
17 differing species.

18 Now as we move to human cell substrates,
19 we will no longer be protected by the species barrier.

20 Even non-human primates have a sequence similarity to
21 convert human PrP and vice versa.

22 So yes, I think that answers it. The
23 exception to that is of course this new variant agent
24 which we're all frightened about because it doesn't
25 seem to obey species barrier.

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1 DR. ONIONS: David Onions. Could I just
2 ask the converse of the question that you posed for
3 vaccine substrates, which is I think one that has been
4 discussed. That is, the idea of knocking out the PrP
5 gene. We know that PrP mice are viable, so it looks
6 like perhaps an interesting way to go.

7 But can you also engineer cells over

8 expressing normal PrP and use those as substrates for
9 infectivity? You mentioned that one of the problems
10 was the low level of PrPC in most of the cell lines
11 you have used. Can you not over-engineer cells so
12 that they become susceptible?

13 DR. CASHMAN: The only—that's never
14 been done. The only data that really pertains is
15 transgenic mice. In fact, if you have knocked out the
16 prion gene, you are absolutely resistant. If you have
17 one normal copy, you have disease with a longer
18 incubation and a shorter rate of—or a longer rate
19 of progression. If you have two normal copies, you
20 have disease at the normal time. If you have
21 transgenic 10, 20, 100 copies, then the disease
22 presents at an earlier age and is more rapidly
23 progressive.

24 As far as I know, nobody has moved that
25 observation to an in vitro paradigm.

1 DR. LOEWER: Johannes Loewer, Frankfurt.

2 I would like to challenge, to some extent, your
3 calculation on the risk of mutations, because they do
4 not take into account the similar biologies of prion
5 protein that are secreted from the cell, as they can
6 spread to other cells. That there's really a short
7 time induction of new PrP Sc.

8 For example, at least to my knowledge, if
9 you infect so to say cell cultures who use prion via
10 PrP, you get another multiplication of infectivity.
11 It stays more or less. If it disappears, it may be
12 more or less there's no real spread.

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

19 DR. CASHMAN: I would very much appreciate
20 some challenge from my numbers. They are new numbers,
21 so don't hold back.

22 The question of spiking, there have been
23 experiments performed at least with a purification of
24 albumin and other plasma proteins. Paul Brown and
25 Robert Roewer published an article last fall in

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1 Transfusion detailing the amount of infectivity if you
2 start from a high amount in whole blood, what you end
3 up with in albumin. In fact, there's at least a 10 to
4 the 6th loss of prion infectivity if one follows the
5 normal protocols for purification of albumin.

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

14 Did that answer your question? I have a
15 feeling it didn't.

16 DR. FRIED: Mike Fried. I think your
17 numbers also don't take into account modifier genes,
18 which could be just random mutation.

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

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

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1 everyone—well, there's a nice distribution in the
2 normal population, about 50 percent are heterozygotes
3 and about 25 percent are met met, and 25 percent are
4 val val.

5 In a new variant CJD, everyone who has
6 contracted the disease to date has been met met. But
7 that in fact may be due to the fact that the met mets
8 are more susceptible to the agent. It may not be due

9 to the fact that val vals or heterozygotes are
10 resistant.

11 DR. FRIED: Sure. I am just saying that
12 that goes into your numbers, whether there's
13 modifications.

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

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

21 CHAIRPERSON RABINOVICH: Modifier genes.

22 DR. CASHMAN: Modifier genes, yes.

23 Modifier genes have of course been proposed from
24 animal studies of infectibility and experimental
25 scrapie. Dr. Prussiner has hypothesized a protein X,

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1 which may be a receptor or may be a chaperon that

2 somehow modifies susceptibility of an animal to prion

3 diseases. There is also a protein Y that Dr.

4 Prussiner has hypothesized.

5 I agree with you from the bottom of my

6 heart that there will be modifier genes affecting

7 susceptibility to prion diseases and the propagation

8 of prion infectivity in vitro. But we don't know what

9 they are yet. At the crude operational level of being

10 able to infect cells, yes. We can infect cells in

11 vitro. So at least some of those modifiers have to be

12 there. Did that make sense?

13 CHAIRPERSON RABINOVICH: Dr. Hayflick,

14 final question.

15 DR. HAYFLICK: Hayflick, UCSF. I was

16 intrigued by your observation that the species barrier

17 for prion transmission is less, is reduced between

18 non-human primates and humans, which would raise some

19 additional concern about the use of primary tissue,

20 and particular, primary monkey kidney tissue for the

21 production of human virus vaccines, because contrary

22 to popular belief, that tissue and any primary tissue

23 does not contain—and I'll use primary monkey kidney
24 as an example, only cells that are derived from a
25 particular part of the kidney.

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1 A primary monkey kidney culture consists
2 of an enormous variety of differentiated cell types
3 that compose the vascular system and neurons. So that
4 monkey neurons do play a part in the production of
5 polio virus, for example, derived from monkey kidney.

6 So that I think that it's important to mention that
7 neurons are not only a part of brain tissue in
8 considering various cell substrates.

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

15 DR. CASHMAN: Just blanking out. So thank
16 you for adding that to the list.

17 DR. HAYFLICK: The final question I have
18 is I may have misunderstood your slide in which you
19 were addressing the question of utilization of human
20 cell line, that a human cell line had not been used
21 for the production of enormous numbers of doses of
22 vaccine, for example, in order to support one of your
23 contentions.

24 Did you mean it in that respect, a human
25 cell line that is defined to be immortal and

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1 transformed? Or did you mean any human cell
2 population?

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

7 DR. HAYFLICK: No. Not as you have
8 defined that cell population. I was interested
9 particularly in normal human cell populations, which
10 have been used for the production of up to a billion,

11 with a B, doses of virus vaccine. But you are not
12 including in that?

13 DR. CASHMAN: Say it again. I'm sorry.

14 DR. HAYFLICK: Normal human cell
15 populations have been used for the production of about
16 three-quarters of a billion doses of human virus
17 vaccine world wide, but these are not continuously
18 propagable abnormal heteroploid cell populations.
19 These are normal finite lifetime cell populations.

20 DR. CASHMAN: So these are cell strains?

21 DR. HAYFLICK: Yes, as I defined them. I
22 realize there's a problem in understanding these
23 terms.

24 DR. CASHMAN: Which vaccines?

25 DR. HAYFLICK: Virtually all pediatric

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1 vaccines, polio, rubella, mumps, measles, rabies,
2 adenovirus, some rhinovirus vaccines, are all produced
3 on a semi-continuous human diploid cell strain like
4 WI38 or MRC5.

5 DR. CASHMAN: Thank you.

6 CHAIRPERSON RABINOVICH: I think you
7 better clarify.

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

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

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

22 (Laughter.)

23 Brilliant as this panel are, I don't think
24 they could do that. So could I have some guidance on

25 when you would like to close this panel session?

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1 Perhaps somebody could give me some guidance.

2 CHAIRPERSON RABINOVICH: Forty five

3 minutes.

4 DR. ONIONS: Forty five minutes. Thank

5 you very much.

6 Okay. We were charged in this panel to

7 answer a number of questions. I'll come to those and

8 try and go through and cover the areas that the panel

9 will discuss. I would be very grateful for as much

10 participation from the audience as possible.

11 I thought it was just useful to pick up

12 two strands that I think came out of some of the

13 comments yesterday. One I think that's important to

14 make, and that is that vaccine production is a very

15 pragmatic process, and that once there have been lots

16 of theoretical objections to particular cell

17 substrates, particularly autogenic cell substrates,

18 there are very practical reasons for the use of cell

19 substrates that might be immortalized or neoplastic
20 from a new generation of vaccines. I don't think we
21 should lose sight of that. There are very practical
22 reasons in scale up and use that I think we should
23 bear in mind to produce therapeutic vaccines.

24 The second concern was that came out, and
25 perhaps didn't get enough airing as it should have

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1 done, and that is there clearly is a trend in society
2 at the moment about concern in safety of vaccines.
3 That perhaps therefore focuses particularly on the
4 item we are going to discuss today, which is the
5 possibility of adventitious agents.

6 The question that the FDA asked us to
7 evaluate, or at least one of the first questions, and
8 they would like the panel to take a look at is do
9 neoplastic cells represent the greater equivalent or
10 lesser risk for the presence of adventitious agents
11 than primary cells, diploid cells, or non chunogenic
12 continuous cell lines.

13 I am not sure, given my previous comments,
14 whether I think necessarily that you can answer that
15 in a simple sense, but does anyone in the panel want
16 to sort of pick that one up first of all?

17 What I could perhaps do is to prompt
18 people, is perhaps to put up those which is just my
19 suggestion, of some of the factors that might
20 influence the risk of adventitious agent testing in a
21 variety of cell substrates.

22 DR. MINOR: I am personally convinced that
23 they are going to be better than primary cultures. I
24 am not sure whether they will be necessarily better
25 than human diploid cells or anything else if we even

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1 get a decent banking system going. It seems to me
2 that when you get to that kind of stage, it's the
3 concern about how you find what's in there rather than
4 anything else. I think the actual extent to which you
5 can characterize them is clearly to me, it's very
6 similar.

7 DR. ONIONS: That would be a generally
8 universal statement that primary cells are likely to
9 be more difficult to characterize and therefore, if
10 you can use a cell line, that is probably the way to
11 go.

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

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

22 DR. ROBERTSON: Another point which one
23 could consider. Where neoplastic cells might be
24 considered more susceptible than primary cell
25 cultures, in that the primary cells are derived

1 specifically for vaccine production. Whereas the
2 neoplastic cells have probably been kicking around at
3 least one, if not several laboratories, before being
4 put into use as manufacturing of vaccines.

5 Because of that, they may well have picked
6 up something that you don't want to be there. Nothing
7 to do with the cell type or the origin of the cell.
8 So a virus of some different species all together
9 which you really have got to check for.

10 So if you are actually setting up a cell
11 bank of a neoplastic cell, you shouldn't just be
12 considering species of origin of that particular cell,
13 whether it's porcine, human, murine whatever. You
14 have really got to consider any virus under the sun.
15 We know there have been instances of this happening.

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

20 DR. ONIONS: There is a good example in a

21 commercial product. That of course was the Glaxo
22 Wellcome's novalma cell line which was used to produce
23 interferon, which in fact contained SNRV, and probably
24 picked up SNRV in George Kahn's lab at some point
25 during its history, I think was the general consensus.

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

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

14 For instance, we have known that in

15 certain tumor cell lines, that viruses that we have
16 only recognized in the last decade are certainly
17 found. For instance, well HHV-6 isn't a transforming
18 virus, but there are cell lines that carry HHV-6 that
19 have been used in the lab for many years. The same is
20 true for HHV-8, which is a transforming virus.

21 So there is a concern that we may have
22 cell substrates that are contaminated by other tumor
23 viruses.

24 Tom, would you like to pick up?

25 DR. BROKER: I think we have actually a

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1 wonderful opportunity for a so-called natural
2 experiment. That is solid organ transplantation. It
3 turns out, as we all know now, virtually all kinds of
4 organs, not only the corneal we have just heard about,
5 but kidney, liver, pancreas, part of the intestine,
6 heart, lung, so forth, have all been transplanted. I
7 think the opportunity is that the recipient is
8 invariably immuno-suppressed until the transplant

9 takes, and then they are slowly weaned off the drug
10 like cyclosporin.

11 Yet on other occasions, the transplant
12 fails for one or another reason. One could go back
13 into failed transplants to look for the reactivation
14 of agents that came from all these different tumor or
15 tissue types I mean.

16 One example I could cite that we recently
17 encountered in the course of our kidney transplant
18 study is a pair of kidneys that went in from a five-
19 year-old boy to a 19-year old female. Within a few
20 days, the kidneys had completely become destroyed,
21 necrotic.

22 It turned out—they suspected CMV
23 infection, but it turned out to be adenovirus. The
24 presumption, and I'm being completely hypothetical, is
25 the five-year-old boy who had died in a bicycle

1 accident, the donor, probably was in the age bracket

2 where adeno was just a natural infection in his
3 airway, and that these cells say from his tonsils or
4 adenoids, which were in the midst of processing the
5 adeno, became circulating, were in the kidneys, and
6 the recipient female then acquired adeno-infected
7 kidneys, and upon transplant to her, the virus
8 reactivated and just wiped out the tissues. I might
9 also say the different individual who received the
10 boy's liver also lost the liver.

11 So presumably these were entering through
12 B cells that were in any of these remote organs.

13 Nonetheless, the basic opportunity to look at organ
14 recipients I think is the experiment to ask how much
15 infectious agent is being transferred.

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

23 infection.

24 DR. BROKER: Well, in that case, it's why

25 I did also point out that the liver who went into a

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1 completely different boy also wiped out. But I agree

2 with you. One doesn't know whether it's endogenous,

3 but it does give some indication of infectious agents

4 in these organs.

5 DR. ONIONS: Could I just actually take a

6 backup actually? I was about to go back to the

7 primary cell issue again. Phil gave I thought a

8 wonderful presentation. I hadn't heard parts of this

9 before. It actually started to worry me a little bit

10 actually.

11 To what extent do you need now to control

12 the kinds of colonies of these particular primary

13 colonies? I'm not sure, I mean I don't know what kind

14 of testing goes on in these colonies for a range of

15 adventitious agents.

16 Can you maybe just comment on that? I
17 mean are we dealing with inverted SPF animals?

18 DR. MINOR: Well with respect to the
19 primate, you will certainly not. But they are

20 increasingly heavily monitored. It depends very much
21 on the manufacturer and how much monitoring they do.

22 One manufacturer, for example, has only

23 recently, well in the last four or five years I guess,
24 started using colonies of monkeys that were monitored
25 for foamy virus. The result of that is being

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1 revolutionary in terms of the number of cultures that
2 you get surviving to production.

3 You would have thought you might have
4 started this a bit earlier perhaps. But you couldn't
5 call them SPF, but they are increasingly closely

6 monitored I think. Certainly some manufacturers have
7 them more closely monitored than others.

8 But one of the difficulties with the whole

9 of adventitious agent business of course is you only

10 really find what you are looking for. That's an
11 ongoing problem.

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

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

25 Could I move to perhaps the third element.

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1 That is, we have heard a lot about retrovirus.
2 Retroviruses always come back to focus when we deal
3 with cell substrates. What is the panel and the

4 group's feeling here in general about the concerns of
5 using either immortalized cells or transformed cells,
6 because frequently those—well, that's not an
7 accurate statement. Activation of transcription of
8 endogenous genes is more frequent in such cells. Is
9 that of concern or not a concern? Or do we have to go
10 cell by cell, species by species, to answer that
11 question?

12 John, would you like to make a comment?

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

19 It is not entirely clear to me whether
20 this actually represents activation of transcription
21 of these cell lines or a fixation of a differentiated
22 state which itself is what's activating the
23 transcription. I am inclined to think probably both

24 are true in different cases.

25 One of the things that we have learned, at

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1 least from human tumor cell lines, is that none of
2 these things that are activated have ever been shown
3 to be infectious, despite the fact that as came out in
4 the earlier talk, the probability for recombination
5 between a large variety, for example of the HERV-K
6 sequences, would seem to be rather high. If these
7 were other types of retroviruses, such as MLVs where
8 you do get that endogenous, you do get that kind of
9 thing.

10 So there's probably something else that's
11 protecting the people in the cell against actual
12 infectivity in this particular case. We don't yet
13 know what it is.

14 DR. ONIONS: I was just going to bring in,
15 I was very struck by the HERV-K story. That is to a
16 very pragmatic level. I take the point that it does
17 seem to me that it doesn't much recombination or much

18 adjustment to me, at least into a more functional
19 virus. I mean should we simply be screening any human
20 cell substrate for the expression HERV-K? Would that
21 be something that would be useful to do?

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

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1 HERV-K family.

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

5 DR. PALLEY: We do not know whether that
6 particular locus is expressed in some cell lines. We
7 know that if this, at least this HERV-K family, is
8 activated for some reason, that there is very likely
9 not only one locus activated within the genome, but
10 that there are several loci that will be activated.
11 So it's possible that this locus is also activated,

12 but we also then have the problem that if that locus
13 would not be activated, that there's some
14 transcomplementation. So one gag, intact gag gene
15 would complement another intact pol gene and so on.

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

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

23 AUDIENCE MEMBER: I think we have to focus
24 on infectivity here, because if we just go looking for
25 expression of defective stuff, you always find it, and

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1 we all go home and won't be able to do anything. It
2 might not be a bad idea perhaps, but --

3 (Laughter.)

4 AUDIENCE MEMBER: Sounds good to me. But
5 just to push this point a little bit further on the

6 HERV-K, you know, about two-and-a-half years ago,
7 there was a report in Cell, which is a better journal
8 than I usually publish in, which claimed that HERV-K
9 env can act as a super antigen that then stimulates
10 diabetes mellitus in some people.

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

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

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

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

25 DR. ONIONS: Can I maybe get your opinion?

1 I really would like to get some feeling because we
2 have heard a lot, and it's scientifically really
3 interesting by the expression of these human
4 endogenous retroviruses. I think John has probably
5 just summarized it. It looks like we're saying the
6 list, this collected group here of retro, are saying
7 that as far as we are aware, at the moment these are
8 not of concern and uninfectious, and probably
9 therefore there is not a great deal of point in
10 looking for expression of these in cell substrates,
11 the very pragmatic practical point.

12 Would that be your opinion too, Johannes?

13 Johannes is nodding. That's a "yes," I
14 assume.

15 AUDIENCE MEMBER: From a research
16 standpoint, it's absolutely worth pursuing to see if
17 one can find these things eventually. But in terms of
18 vaccine issues, I don't see how we could possibly deal
19 with it now.

20 DR. SCHUEPBACH: Yes. I also would like
21 to make a comment regarding that super antigen
22 activity because we are coauthors in that paper. It
23 is true that the presence of these sequences, RNA
24 sequences in the serum we can not repeat, so it's not
25 specific for IDDM patients. But to my knowledge, our

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1 data regarding the super antigen activity and the
2 stimulation of VP cells has not been disputed by any
3 other group. So that is still around.
4 I think that the real important topic here
5 is whether these endogenous viruses actually give rise
6 to infectious particles. I believe that with the PERT
7 assay, we actually can contribute very much to this
8 question. I think, as I pointed out, of course the
9 easiest thing is to test the super natants for
10 reversed incriptase activity. But I think with a
11 little bit of additional work, it should also be
12 possible to define that profile of RT activity and
13 cellular, DNA polymerase activities along the

14 different fractions of the sucrose gradient and tend
15 to recognize any abnormal pattern that might be
16 associated with infectious rate of viruses.

17 AUDIENCE MEMBER: Let me just go back then
18 because obviously the question comes up if the public
19 is asked to accept a vaccine that's made in cells that
20 express HERV-K. Even if one part of the story has
21 been refuted, the question comes up. You know, are
22 there potential immunological consequences of the
23 expression of antigens from these kinds of cells which
24 are not expressed in human diploid cells? Even if
25 this story is wrong, I guess, and it sounds like it

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1 hasn't all been refuted, the question then comes up of
2 what level of scientific data in the literature is
3 necessary to completely refute it?

4 I can imagine the outcry that could occur
5 if people believe the story. If there appears to be
6 some controversy about what part of this story has
7 been refuted, then I think one might have a public

8 confidence problem as well.

9 DR. HENEINE: David, I have a comment
10 which could be redundant, but go ahead and say it.
11 While thinking about all these questions from my
12 simple mind, it looks like if you want to compare cell
13 lines versus primary or diploid cells, the two
14 questions that were raised is which ones transmit less
15 adventitious agents or transmit less neoplasms to
16 vaccine recipients.

17 What we have heard so far about the
18 mechanisms of the neoplasms, many of those are
19 mediated by viruses or viral-like elements. So it
20 looks to me that the majority of the concerns are
21 rising from the adventitious agent group rather than
22 from other elements.

23 So therefore, in trying to make up our
24 mind, based on the available data, which one is the
25 more suitable substrate, maybe we can go very simply

1 with a checkpoint list on these different cells,

2 targets, which one we can test for the presence of
3 these adventitious agents known, unknown, and which
4 can be better monitored, which can be for practical
5 reasons of culture as well, and make up our mind,
6 rather than jumping right and left with different
7 issues.

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

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

22 So I think the only thing that matters is
23 endogenous retroviruses from other species, not from
24 humans.

25 DR. LEWIS: This is a question. I think

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1 we are sort of being faced with something of a dilemma
2 here, because as you very correctly pointed out, the
3 one way to look for a retro virus, adventitious retro
4 viruses or endogenous retro virus, whatever, is by the
5 PERT assay. Now if we take a situation in which our
6 HERV-K is expressed and there's RT activity in there
7 with the PERT assay, from a regulatory perspective,
8 what do you do about that?

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

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

16 is positive?

17 DR. SCHUEPBACH: I think once you have

18 activity, then you have to characterize what it is.

19 Depending on whether this is exogenous or endogenous

20 virus, steps will be taken. I think identification is

21 important.

22 DR. LOEWER: I think we shouldn't continue

23 to discuss use of this type of cell lines in absolute

24 terms, because the other side of the coin of course is

25 a product which could be made from it. This is always

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1 to be judged in conjunction, in my opinion. For

2 example, there's the question of endogenous sequences

3 which may be active, just retro virus or line

4 elements. So far as I know, this is not the same in

5 all cell lines. It's mainly expressed in tumor cell

6 lines, and so far as I know, there is so far no need

7 to use these cell lines for vaccine production.

8 If somebody believes it's necessary, must

9 be a very special virus which can only replicate in
10 these cells, then perhaps we do not have another
11 choice to use them.

12 DR. ONIONS: Could I just go to Arifa?

13 She has been waiting very patiently. Then Steve.

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

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1 find at some level in all human cells.

2 DR. ONIONS: I would like to endorse that.

3 I think really we're making it too complicated. I
4 think it's very simple. You use that kind of assay
5 system which has a very high sensitivity developed by
6 Yumascript here in the audience. Then if you get a
7 positive, then you go and look and see if there's
8 something there that's infectious. It is a hierarchy
9 of testing strategies, it seems to me.

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

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

21 So the obvious criteria is not whether or
22 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

17 get a more sensitive technique, maybe you are correct.

18 But there is a very strong distinction if
19 you look at cell lines like MRC-5 and compare them to
20 what you see in say AB, you get signals, if you're
21 looking at MRC-5 you don't. If you look in tetra
22 anacells, you get a signal.

23 DR. HUGHES: I would argue that is with a
24 particular assay that has been tuned up to detect RT
25 in a particular way. I would be willing to wager that

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1 if we look hard enough, we could certainly find
2 evidence of particle production in any of these cells,
3 simply because they are full of endogenous viruses
4 that—I mean the very fact that there's obviously
5 expression is RNA present.

6 DR. SCHUEPBACH: But the important thing
7 is I think the number of particles. You have very,
8 very low number of particles, and you do a test, a
9 PERT assay from a concentrated material where you
10 pellet virus, let's say, from one liter, and assay

11 that and find a very low activity. You know for
12 production of the viral vaccine, the cell harvest, the
13 vaccine harvest will be diluted 50 to 100. You also
14 realize that most of the particles even of infectious
15 retroviruses are non-infectious. Then you certainly
16 can come to some calculation which permits you to
17 establish a level of safety where you have a very high
18 probability that this vaccine is safe.

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

23 If the particles are infectious, a very
24 small number is very important. If the particles are
25 not infectious, in particular, if the particles are

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1 not infectious for humans, the presence of a
2 relatively large number of particles is probably also
3 irrelevant.

4 DR. ONIONS: My only caution about that is

5 that you can make assumptions about infectivity that

6 also are not true. Since Clive Patience is here --

7 DR. HUGHES: I think you have to do the
8 test. I don't think you can make assumptions about

9 it.

10 DR. ONIONS: Well, the point I was going
11 to make actually was that Clive's group and our group
12 showed that you could actually infect cells with PERV,
13 yet those experiments have been done 20 years ago, and
14 been done by very good people, including George Tadaï,
15 and were unable to show infectivity. It's just the
16 techniques have changed slightly and we could get
17 infectivity.

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

21 DR. HUGHES: I am not trying to suggest
22 that the assays that we have for infectivity are
23 necessarily always 100 percent accurate. But what I
24 want to get away from is the idea that the presence of

25 a physical particle is somehow a measure of safety or

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1 lack of safety.

2 I certainly agree with you that there are
3 numerous technical problems in determining
4 infectivity. But what I believe we should focus on is
5 better ways of doing infectivity assays rather than
6 better ways of doing physical assays.

7 The physical assays can be very useful if
8 they are coupled to infectivity. In fact, I believe
9 that was a statement that John Coffin made. What I
10 would propose is what I think John Coffin proposed,
11 that we actually use these really wonderful sensitive
12 techniques, but in the context of measuring whether or
13 not the viruses are infectious, not whether the
14 particles are physically present.

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

17 DR. HUGHES: Sure.

18 DR. SHEETS: I'm Becky Sheets, FDA. What

19 I hear you suggesting is that rather than testing for
20 RT activity by a physical assay, as you called it, a
21 PERT assay or a conventional RT assay, you think that
22 it would be preferable to test vaccines for
23 infectivity assays?

24 DR. HUGHES: The ability to transmit that
25 RT assay to a reasonable recipient cell. I believe

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1 this is exactly what John --

2 DR. SHEETS: The pragmatic question is
3 that would you do this testing lot by lot on vaccines?
4 For instance, if you were making a vaccine in a
5 primary cell substrate, for instance an egg, would you
6 test each lot of vaccine or each batch of vaccine for
7 infectivity assays? Then the really pragmatic part of
8 it is, if you are making a flu vaccine, where the
9 timing of production, the timing of testing, and the
10 timing of lot release is very tight, would you
11 recommend these infectivity assays on lot by lot for
12 primary source?

13 DR. ONIONS: We're running out of time.
14 Do you want to answer that? You have been asked a
15 question, do you test lot by lot?

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

24 DR. SHEETS: That's fine for SPF
25 situations. I am asking this question because this is

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1 what sponsors ask FDA. So they want to know do we
2 need to do this lot by lot, or if a cell bank, you can
3 do a one-time characterization. Of a primary system,
4 you can't do it that way.

5 DR. HUGHES: Use SPF chickens and don't
6 ask.

7 (Laughter.)

8 DR. LOEWER: I would like to make a

9 comment to Dr. Hughes' comments. They are very sound

10 in a scientific meaning, but they face regulatory

11 problems, the main problem indeed.

12 Regulatory authorities have to show that

13 there is no infectivity and the proof of non-activity

14 is always nearly impossible in a scientific sense.

15 You will always find reasons to say you were not able

16 to find infectivity. Look at HIV. If you would use

17 MRC-5, for example, or a lot of other animal cells,

18 you would never find infectivity of HIV. The same is

19 true for many situations.

20 So there a fundamental problem is to test

21 for non-activity or noninfectivity.

22 DR. ONIONS: I would like to stop this

23 because we are running out of time and there are other

24 issues.

25 I am going to take the Chairman's

1 privilege and just say that I actually think you need
2 multiple techniques, because I think as Johannes has
3 just said, if you have complete infectivity, you will
4 miss things as we would have missed cell lines
5 producing ver. I think you really need to have a
6 combination technique. So I think it's a belt and
7 braces situation. That's a personal view.
8 What I would like to move onto, is were
9 asked by the FDA also to consider species of origin.
10 I think really you end up in very general statements
11 here. You can argue that if you are worried about
12 adventitious agents, then clearly there are species
13 barriers to the transmission of some agents. On the
14 other hand, other agents do go across species
15 barriers, sometimes in abortive replication. They can
16 be very nasty. We of course know that herpes B going
17 across species barriers is actually lethal. Ad 12 in
18 hamsters is oncogenic. Equine herpes veras, which is
19 an alpha herpes veras is oncogenic in hamsters. There
20 are natural examples of cross-species transmission,

21 the ovine herpes veras 2 is innocuous in sheep, but it
22 kills cattle. So there are examples of these
23 heterotic transmissions being worse than natural
24 infections.

25 Is there anything that we can say, the FDA

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1 have asked us, in a general statement about species of
2 origin? My own view is I don't think you can, but
3 does anyone want to make a statement?

4 DR. MINOR: I think sometimes it is better
5 and sometimes it's worse.

6 (Laughter.)

7 DR. ONIONS: Yes. That's exactly what I
8 think. Thank you, Phil.

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

13 I don't want to trespass really on
14 yesterday's, but I think maybe just to come back to --

15 we're going to move onto assay systems in a second,
16 but I think one of the issues we're coming around to
17 in a second is latent viruses, because those seem to
18 be the real concern. It may be worth just remembering
19 some of the things that were partly discussed over the
20 last two days. That is, that the complementation of
21 defective viruses can occur. For instance,
22 adenoarectus can be complemented by HPV in hela cells.

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

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1 lot about retrovirus retrovirus pseudotype formation,
2 but this can occur across viral species. For
3 instance, paramyxovirus is rather badly, but they can,
4 pseudo type retro viruses. So you could alter the
5 host range when endogenous agent is expressed in your
6 cells.

7 Of course there are recombinants. Some of
8 these recombinants, and we have got representatives

9 who did the work here, interesting recombinants like
10 SV-40 adnivos recombinants.

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

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

21 Anyone want to comment on what we should
22 be doing about novel cell substrates and you have got
23 a virus there that you don't know anything about.
24 What sort of technique should we be applying?

25 DR. PALLEY: I would like to make a

1 comment from that HERV field. I think if we talk

2 about possible recombinations of HERVs with some other
3 viruses that might be a little difficult, I think it
4 could be conceivable.

5 We do not know, however, it's very hard to

6 predict the outcome of whether it is possible at all.

7 We basically have or we might have situations where a

8 HERV is expressed where another virus, retrovirus is

9 also present in the same cell. I think that has to be

10 discussed or taken into consideration, that there

11 might be possible recombinations between HERVs and

12 some retrovirus status put into a particular cell.

13 However, I think it's very hard to predict whether

14 there's any possible recombination and what the

15 outcome of that recombination might be.

16 But I think there are examples where retro

17 viruses indeed recombine with each other and produce

18 some productive outcome. But it's very hard to

19 predict what, in which ways HERV case for instance, or

20 whatever HERV sequence within human genome could

21 recombine with something else.

22 DR. ONIONS: I was just going to point out

23 I think one of the things I have been very struck by
24 by some of the talks here, particularly the
25 polymerized talks, it does strike me, the comment I

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1 made to Phil earlier about the use of cynomologous
2 monkeys and perhaps have these been screened for other
3 polyoma virus. That seems to be of concern. We know
4 that polyoma viruses are coming in as contaminants in
5 bovine serum into the primate cells, the bovine
6 polyoma virus. They worry me as potential
7 adventitious agents. I am just wondering again
8 whether we should be doing more in terms of redundant
9 PCR approaches to look for these agents in both
10 primate and non-primate cell lines.

11 Any views on that?

12 DR. ROBERTSON: This is potentially the
13 most important door, also the most difficult to deal
14 with. If you think back to what Phil was saying in
15 the first talk this morning, all these instances of

16 vital contamination, generally they occur with viruses
17 unknown at the time, viruses expected, the presence of
18 viruses in vaccines or biological preparations.

19 Potentially is not something we're talking
20 about today, it's not an endogenous virus or
21 recombination between an endogenous virus, but
22 something unknown that's going to leap up at us out of
23 the dark. Of course almost impossible to deal with.

24 But Joerg was saying this morning about
25 this is what we should be looking out for, the

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1 unknown, and if possible using a more broadly reactive
2 type of assay rather than highly specific type of
3 assay to look for something.

4 If we knew to look for something, that's
5 fine, we can deal with it. It's what's not there
6 which causes the problem.

7 DR. SCHUEPBACH: May I add something to
8 this? I think our chances to detect such unknown
9 agents are really much better if they are present at

10 high concentration. So I don't know whether you
11 accept that concept of cellular cloning in order to
12 either get rid of these agents or to have them at the
13 very high concentrations so that their detection is
14 actually much easier. In the meantime, you can try to
15 activate the host cells by all kinds of different
16 agents. You do EM studies, you do serological
17 studies, use broadly cross-reactive antibodies. I
18 mean this is a wide field actually of methods you can
19 employ.

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

23 DR. MINOR: Can I ask for a definition
24 here? What do you actually mean by a virus? I mean
25 what I would understand by a virus is something that

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1 actually grows in the cell and increases in numbers.
2 If you think back to the very, very early days of
3 polio vaccines, amongst others, there was an awful lot

4 of effort put into trying to make sure that you put
5 your supernatant or whatever, into all sorts of
6 potential different systems where a virus might grow.

7 So you put it into different cell
8 substrates and you look for cytopathic effect, where
9 you can say maybe you have got a virus that doesn't
10 cause a cytopathic effect. You put it into mice, you
11 put it into eggs, you put it into everything. So it's
12 like evidence of actual growth. You see?

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

21 DR. ONIONS: Well, okay. Let me give you
22 an example. I think one of the areas that is going to
23 emerge as a concern are going to be circaviruses. The

24 reason I say that is that TGV may be a circavirus. We
25 now know of others. Thomas was talking about the kind

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1 of normal flora of HPVs. It looks like we all have a
2 normal flora of these circaviruses. Certainly when we
3 started now looking in animals, we find these all over
4 the place.

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

9 What we know is if you look in a cell
10 substrate like PK-15, which has used in the pig
11 vaccine industry, that carries a circovirus genome.
12 It looks, it's possible semi-defective, but you can
13 introduce it as an infectious agent, but most of the
14 time it isn't inducible as an infectious agent.

15 So I think I'm not quite sure I take your
16 distinction. I think there are latent viruses there
17 that are reactivatable under certain circumstances and

18 are a concern. In fact, in the vetmurines vaccine,
19 you have to get rid of that virus.

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

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

3 Very briefly, what's done is the L-1 and
4 L-2 capsid proteins of papilloma virus self assemble
5 no matter where they are over produced. Four
6 different groups are pursuing these now. I am sure
7 they are engaged in some corporate relations.

8 But one of the neat strategies that John
9 Shiller here at NIH and others have done, is to fuse
10 peptide epitopes or even intact other genes to the L-2
11 protein at their n-terminus. It turns out that when

12 L-2 assembles into the virus-like particle, but brings
13 this n-terminal protein in with it, that other protein
14 is on the inside of the virus-like particle. So it
15 will ultimately be presented to the recipient of this
16 virus infection. It's non-genetic, but it will be
17 taken up by cells. So there is in effect no risk of
18 some bacteria phage infection running rampant in our
19 bodies. So it's a complete alternative.

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

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1 Could I just ask perhaps questions about
2 we had examples indeed from Thomas toady about using
3 redundant PCR techniques. It would be feasible to
4 screen for a number of viruses that are of concern,
5 like herpes viruses, circoviruses, polyoma viruses,

6 herpes viruses retro viruses by redundant PCR
7 techniques. These are not in FDA terms, validated
8 techniques. On the other hand, they are very powerful
9 techniques. For instance, one of my colleagues just
10 developed a herpes virus redundant PCR technique with
11 112 primer combinations that to date has picked up all
12 of the herpes viruses that's been challenged with both
13 human and animal origin. So you could go looking for
14 herpes viruses by that kind of technique.

15 Robin Weiss' group in London picked up a
16 new human herpes virus, HRV-5 by a redundant PCR
17 technique. So do any of you think that these kinds of
18 technologies should be implied to cell substrates? If
19 so, which viruses should we use? Because it is a lot
20 of work to do this kind of thing. So should we be
21 doing this or is it not necessary? Can I have
22 comments?

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

25 DR. ROBERTSON: One would not expect to

1 see this type of assay risen up in a pharmacopeial
2 recommendation of any kind. But certainly they do
3 have uses at the investigational level, especially
4 with some incredibly novel cell types.
5 Everything is kind of going molecular
6 these days. All these assays are looking at things
7 from the very molecular point of view, and picking out
8 a signal say with your herpes primers, need not
9 necessarily say that you have got an infectious herpes
10 virus.
11 So go back to this argument we have been
12 having this morning about it's infectivity which
13 potentially is what we're concerned about, and not
14 picking up a fragment of a genome.
15 These are assays, again I mentioned that
16 Joerg mentioned them this morning, broadly reactive
17 molecular assays. Phil rightly pointed out that we
18 have had in place for eons broadly reactive
19 infectivity assays using tissue cultures, suckling

20 mice eggs, looking for signs of infectivity of any

21 kind.

22 DR. ONIONS: But don't you think—my

23 feeling about that is, I tend to share, I don't think

24 one would ever use these kind of assays on a routine

25 basis.

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1 DR. ROBERTSON: No.

2 DR. ONIONS: They might be useful in

3 establishment of a master cell bank or something, you

4 know, the first one. But I mean I would criticize, I

5 don't think that current infective assays do pick up

6 everything. I think that's the whole problem. I

7 think, for instance, that it would miss—well,

8 polyem virus has perhaps been used.

9 DR. ROBERTSON: At the end of the day,

10 that's the weakness of anything, that you will not

11 pick up something that's not designed to pick up. You

12 will potentially miss viruses in an infectivity assay.

13 But you also potentially miss viruses with a redundant

14 PCR. If you don't pick something up, you can't say
15 it's there or not there. It's a bit of a
16 philosophical argument.

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

22 DR. ROBERTSON: Sure. Oh yes.

23 DR. ONIONS: Does it have anything to do
24 with the—I mean there are people out there who have
25 to do this for a living, rather than us who can just

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1 sit here and pontificate about it. How does this go
2 down with the industry? What does the industry feel?

3 DR. PALLEY: Just one point regarding
4 again HERVs.

5 DR. ONIONS: Sorry?

6 DR. PALLEY: I mentioned in my talk that
7 HERV-W family that has been reported for the first

8 time this year and has been isolated from vitro by
9 particles from multiple sclerosis patients. It turned
10 out that it at least codes for an env gene. So it's
11 certainly worth—the human genome I guess is among
12 the genomes that regarding endogenous retroviruses is
13 among the best characterized genomes besides mouse,
14 for instance.

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

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

1 for hepatitis B in blood and blood products. There is

2 always concern about what are you going to find, and
3 what are the implications, and what are the costs, and
4 all of those things.

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

18 Those things, you can't sit here in a
19 meeting or on a panel and give specific answers to
20 those questions. I think the bottom line is as
21 technology evolves, and it's going to continue to

22 evolve with more sensitivity and specificity
23 hopefully, it needs to be explored and it will find
24 its appropriate place in the overall testing, whether
25 it's in characterization or perhaps in some cases, if

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1 it's appropriate, on a routine basis.

2 But I think the general principle of
3 applying new technology as it begins to be available
4 to look at these issues, particularly as they relate
5 to safety and the presence of adventitious agents, is
6 unassailable.

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

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

12 AUDIENCE MEMBER: I agree completely with
13 what John says, but would add that if we are faced
14 with a decision of whether to approve the use of
15 different types of cell substrates that are

16 tumorigenic or derived from tumors from which we don't
17 know the mechanism of transformation, we are faced
18 with not only the question of should technology be
19 applied, but is the technology as it exists today and
20 can be applied today, good enough to permit us to say
21 that it's okay to use these cells. So that is a very
22 practical question which perhaps could be answered.

23 DR. ONIONS: Can we have a view on that?
24 That is, given the technology we have today, is it
25 acceptable to use the kinds of substrates we have been

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1 talking about, that is tumorigenic or immortalized
2 cell substrates? Are we confident that with the
3 technology we have, that we can use these cell
4 substrates safely?

5 DR. BROKER: I would just basically say I
6 think so. I think if we combine PCR with these
7 microchip or microprobe arrays, DNA chip technologies,
8 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

1 B-lymphocyte might be important to carriage, then

2 perhaps lymphoid cells, particularly B cells that are

3 invariably used, and since we're looking at the
4 possibility of using HIV and T cells, maybe that
5 suddenly becomes an issue. Maybe we should be looking
6 at lymphoid cells for the potential of there being
7 spongiform encephalopathies.

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

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

23 do that in T lymphocytes? It's a trivial thing to do?

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

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

4 DR. ONIONS: I was thinking of excluding
5 the origin of the familial form, which clearly occurs

6 to some inherited disorders. I mean I think you are
7 right. You can't cope with a somatic mutation.

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

11 DR. ONIONS: Neil touched on validation
12 technology. I think that's important. I think there
13 are new techniques for doing validation of TSE
14 removal, but using a disrupted PrP protein. But I
15 don't think that's going to be applicable to quite a
16 lot of the processes that are used to produce vaccines

17 at the moment. It is by technology products, but not
18 to vaccines.

19 I just wanted to touch on testing because
20 I know that Neil has an interest in that area. It
21 seems to me that we were sort of rather optimistic a
22 couple of years ago, and indeed, there have been
23 publications by Bruno Esch and others on specific
24 antisera of PrP Sc. But those haven't held up. They
25 actually pick up aggregated protein and not, as I

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1 understand it, strictly PrP Sc.

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

9 Would you like to comment?

10 DR. CASHMAN: I think that the bio assay,

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

20 DR. ONIONS: Okay. On that optimistic
21 note, I am going to wrap the session up. I think
22 we're all getting edgy for lunch. Unless anyone has
23 got some burning issue that they wish to go into
24 before we go.

25 If not, I think the panel members, and

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1 thank you all for participating.

2 CHAIRPERSON RABINOVICH: Thank you, Dr.
3 Onions. There is a light repast outside for those
4 that have been so patient. I would like to get

5 everybody back in here in 15 minutes.

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

10 Thank you.

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

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

22 The first night I got here, as some will
23 recall, I came in a little late. I ran into a couple
24 of you in getting a beer because it was after the time

25 of the close of the meeting. The discussion ensued as

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1 to what is a designer cell substrate. What do we mean

2 by that? My first reaction to that of course was it's

3 anything that I happened to have made. Clearly at

4 this point in the meeting, it's not primary cells.

5 I suppose from a strictly semantic

6 perspective, it would be a cell substrate created with

7 specific characteristics. It could be immortal or

8 not. But I think over the last couple of days, at

9 least my thinking on this and for the purposes of this

10 discussion, by a designer cell substrate, we mean a

11 cell substrate of defined origin and with a defined

12 pedigree.

13 It is probably immortalized because it is

14 likely to have been cloned. It will be validated as

15 specific pathogen-free and at least specific pathogen

16 sought and perhaps in certain circumstances, defined

17 as non-infectious.

18 For the purposes of the next discussion,

19 we are really talking about immortalization. Jim
20 McDougall, as you know, presented his paper yesterday.
21 So we'll start this session with the first
22 paper by Dr. John Sedivy from Brown University, who
23 will talk about differences in the capacity to
24 immortalize rodent, primate, and human cells by tissue
25 culture passage or viral transformation.

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1 DR. SEDIVY: Thanks very much for the
2 invitation. I am sorry that Jim gave his talk
3 yesterday because—well, maybe it will jive all
4 together.

5 I was asked to give somewhat of a
6 historical overview on the issues of replicated
7 cellular senescence, and obviously the topic of
8 cellular immortalization.

9 So from a historical point of view then,
10 this is the Hayflick phenomenon. This experiment has
11 been performed in numerous labs and always with the
12 same result. This happens to be an experiment in my

13 lab. You will see a number of slides like this from
14 me today. What we're plotting here is replicated
15 lifespan, the doublings of the culture versus days.

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

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

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1 the issue of media and media artifacts, and have these
2 really been adequately resolved today. I don't think
3 they have, especially for some more specialized cell
4 types. I think they have been pretty well resolved
5 for keratinocytes, maybe breast epofelial cells,
6 fibroblasts, et cetera.

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

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

24 The next phenomenon that one often sees
25 cited is the age of the donor plotted against—here

1 is the age of the donor, and the remaining life span
2 of the cells, in this case fibroblasts taken from that
3 donor. As you can see, the points are all over the
4 place. In fact, more recently, this view has been
5 challenged by a recent paper in PNAS from Vince
6 Cristofalos, who actually claims that this correlation
7 doesn't exist. But if you read the literature, you
8 will see this coming up over and over again.

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

15 So this really is the issue here. How do
16 we differentiate between senescence, quiescence, and
17 differentiation. I think that for the purpose of
18 discussion today, this is really not a point of major
19 interest, but for historical reasons I'll go through
20 it rather quickly.

21 Quiescence is defined as a reversible
22 process. So what we are talking about here is
23 essentially a cell cycle phenomenon. That is, we can
24 have a culture that is cycling or contains a large
25 fraction of cycling cells. Then these cells can

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1 withdraw into the quiescence state. Then when they
2 are induced with the proper growth factors, and here
3 of course the key phrase is what are the proper growth
4 factors to elicit this phenomenon. At any rate, we
5 are talking about a reversible process. Whereas
6 senescence by definition is irreversible.

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

15 many cases.

16 So really this has given the impetus to a
17 search for molecular events. So then if we pose the
18 question are there molecular events that are unique to
19 senescence versus quiescence versus differentiation,
20 again, the picture is not very clear cut. I don't
21 want you to absorb this whole slide. Suffice it to
22 say that this is well, not all, but the major part of
23 the regulatory circuitry in G-1. Here you see the D-
24 type cyclance. CDK-4 and CDK-6 driving RB
25 phosphorylation, which in turn drives the second

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1 phase, which is cyclin E production, activation of
2 CDK-2. Of course there are a lot of modifying
3 proteins here, CDK inhibitors, kineses that activate
4 the basal CDK kinase, et cetera.

5 Now this is an area that is receiving a
6 lot of attention. The general theme, at least to me,
7 it seems that there's a high degree of overlap between
8 mechanisms that regulate quiescence, senescence, and

9 differentiation. I don't think this is really
10 surprising because all these three states are
11 characterized by the absence of cell cycle
12 progression. In most cases, by an arrest in the G-1
13 or a G-0 state.

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

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

1 So let me turn to the issue of

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

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

20 This again is the Hayflick plot. What I
21 am showing here is a rodent culture, mouse in this
22 case, and human. This little bump on the curve in

23 fact is senescence for a mouse fibroblast culture. So
24 it's been known for a very long time that rodent cells
25 can overcome senescence spontaneously.

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1 You can also see the great difference
2 between the replicated lifespan in vitro of human
3 cells that go on for a very long time. If this
4 experiment here was continued, it would level off and
5 you would see the typical Hayflick phenomenon. So the
6 human plateau up here in fact is corresponding to this
7 rather short plateau senescence in rodent cells.

8 So the relatively low frequency of
9 immortalization—I should point out that this
10 doesn't really seem like a low frequency, but on a per
11 cell basis, it actually is an event that has a
12 frequency of 10 to the minus 5, to 10 to the minus 6.

13 It's just that the X axis is plotted in days here.

14 The fact that this immortalization can be
15 stimulated by mutagens has led to the hypothesis that

16 this in fact is a mutational event in nature. This is
17 supported by the existence of several viral genes,
18 such as, and we have heard about them here, SV40 large
19 T antigen, polyoma large T antigen, animal virus E1A,
20 HPV E6 and E7, that can cause immortalization. In
21 fact, when these genes are introduced into rodent
22 cells, they are sufficient to cause immortalization in
23 a single step. In other words, if you take a rodent
24 culture and you put SV40 large T into those cells at
25 this point, the curve would look like this. No

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1 apparent senescence under the right culture
2 conditions.
3 So what are these viral oncogenes doing to
4 promote immortalization? Without going into a lot of
5 detail, there is a large body of evidence that now
6 indicates that these proteins interfere with the
7 function of the P53 and/or RB growth inhibitory
8 pathways. In agreement, there's a lot of data from
9 knock-out mice now recently that has shown that the

10 elimination by gene knock-out of a variety of
11 negatively acting effectors can result in apparent
12 one-step immortalization, as shown here for example.

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

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

25 Let me now talk a little bit about the

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1 phenomenon of crisis. So what happens when we put,
2 for example, SV40 large T or E1A into a human
3 fibroblast? What we get instead of immortalization,

4 is a phase of so-called extended lifespan. So here we
5 see a primary cell, the initial proliferative phase.

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

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

20 Senescence is different from crisis.
21 These are not just the same proliferative decline.
22 The main distinction is that cells in senescence or M1
23 are truly non-dividing. Whereas in crisis cultures,

24 the apparent absence of proliferation on the
25 macroscopic scale is actually the result of ongoing

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1 cell division combined with ongoing cell death.

2 This is an experiment that was performed
3 in my lab. What we show here is that elimination of
4 the CDK inhibitor P21 in a pre-senescent normal human
5 fibroblast causes an apparent extension of lifespan
6 that is equivalent in magnitude to that elicited by
7 SV40 large T antigen. So also in human cells now we
8 have been able to do ablative intervention. That is
9 eliminate the activity of certain negatively acting
10 effectors and cause an apparent extension of lifespan.

11 In terms of cell substrate design or the
12 technology that would go into doing this, this was
13 really strictly an aside, we have now developed
14 methods—these are really based on gene knockouts,
15 homologous recombination gene targeting, that can be
16 used to delete entire genes, multiple genes in human
17 cells, including normal human cells.

18 So let me now turn to my last topic, which
19 is the molecular clock of aging. I think probably
20 this is where I am going to overlap with what Jim has
21 already said. As I told you, there are some older
22 observations that correlated entry into senescence
23 with the lap cell division as opposed to chronological
24 time. Quite a few years ago, this has led to the
25 proposal for the existence of some sort of a molecular

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1 clock. Then one envisioned that the running down of
2 this clock would generate a signal that triggered the
3 senescence program.

4 Then the expression, for example, SV40
5 large T could either prevent senescence by overriding
6 a signal from this clock or by what I think is more
7 likely now in light of new evidence, actually
8 interfering with the senescence machinery itself.

9 So as you know, the currently prevailing
10 hypothesis is that the nature of the molecular clock
11 is the attrition of telomeres. This is a slide by one

12 of my dear friends, Chris Counter, who has fancifully
13 imagined H-TERP, which is the catalytic sub-unit of
14 human telomerase sitting here at the end of a
15 chromosome end. So this is a telomere here. Then
16 catalyzing the addition of the telomere heximer. You
17 can see the telomerase RNA that acts as a template for
18 that process right there.

19 Germ cells and some key stem cells are
20 known to express telomerase catalytic activity while
21 the majority of somatic cells lack this activity. The
22 estimation of telomere shortening for one generation
23 is in human cells between 50 to 100 bay spares. So
24 that's 50 to 100 bay spares per S phase. This
25 correlates reasonably well with the average telomere

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1 length in a young human fibroblast of 18 to 20
2 kilobases and the length of 8 to 10 kilobases in the
3 senescent fibroblast.

4 I think it's an important observation that
5 senescent cells in fact contain appreciable

6 telomerase. So here we have a normal cell or a young
7 cell. We get attribution of telomerase. At this
8 point, the telomerase are maybe 8 to 10 kilobases in
9 length. This generates a signal. If the cell is now
10 driven into the extended lifespan phase, these
11 telomeres will continue to erode because telomerase is
12 not expressed in that state. Eventually one enters
13 into a crisis which is caused by erosion at the end,
14 genetic instability, et cetera, et cetera.

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

24 The linguistic definition of senescence is
25 the state of being or the process of becoming old.

1 This term has therefore been used to describe
2 essentially any sort of age-related irreversible
3 proliferative decline. In light of these new
4 molecular insights, I prefer to use senescence in the
5 more restrictive mechanistic sense to designate the
6 response triggered in normal cells. I really believe
7 that senescence is an active genetically programmed
8 process that responds to an inductive signal. Perhaps
9 telomere shortening, but that is not 100 percent
10 clear.

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

20 shortening on many chromosome ends.
21 So now this is really just a restatement
22 of the two-stage mortality process. What I have added
23 here now is telomere length in kilobases on the Y
24 axis, the replicative age on the X axis. So here we
25 have a cell in the beginning. If this happens to be

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1 a germ cell or a stem cell, it will maintain telomeres
2 because it will express telomerase activity.
3 Most somatic cells will start down the
4 slippery slope of telomere attrition, eventually
5 entering into a physiological state of growth arrest,
6 through which they can be driven by either the
7 expression of certain viral oncogene or the ablation
8 of certain inhibitory pathways that are intrinsic to
9 those cells.
10 The cells then enter into extended
11 lifespan. They continue to erode telomeres. They
12 enter into a state of crisis, which is characterized
13 by genomic instability. Finally, at this point, one

14 can attain a truly immortalized derivative in the key
15 step here, is the expression of telomerase catalytic
16 activity.

17 I should also point out that telomerase
18 need not be expressed at the final step. It has been
19 shown experimentally that telomerase can be
20 artificially or experimentally activated anywhere
21 along this line, and that that will lead in some cell
22 types, not necessarily all cell types, to
23 immortalization. However, I think the large body of
24 evidence suggests that at least in vivo, and by this
25 I mean during the natural development of malignancy,

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1 the activation of telomerase activity is a relatively
2 late step.

3 So if crisis doesn't exist in rodent
4 cells, and bypass of senescence is sufficient for
5 immortalization, how does telomerase become expressed
6 in somatic cells, rodent somatic cells? The bottom
7 line here seems to be that telomerase is not very

8 strictly regulated in rodent cells and tissue. A
9 variety of rodent tissues have been shown to express
10 telomerase activity. Telomerase negative primary
11 cultures often become telomerase positive over time
12 even prior to reaching senescence.

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

21 In other words, one step immortalization
22 that one sees so often in rodent cells may in fact
23 require two steps, the obvious step of senescence
24 bypass and very likely a second step that may be very
25 subtle, at least in rodent cultures. That is, of

1 activating telomerase catalytic activity.

2 So I think that is about as good a summary
3 as I can think of in 20 minutes. I will be glad to
4 entertain questions.

5 (Applause.)

6 AUDIENCE MEMBER: Bill Egan, from the FDA.

7 When you immortalize cells, you know, after they go
8 into crisis or whatever, what becomes the length of

9 the telomere? Does it go back up to 20 kilobases?

10 What maintains the length of that telomere at a fixed

11 --

12 DR. SEDIVY: That's a very good question.

13 AUDIENCE MEMBER: Why doesn't it become 30

14 or 40 kilobases.

15 DR. SEDIVY: In fact, it seems that

16 excessive telomere length is not good, at least in

17 human cells. It's been known for a long time that

18 many spontaneously immortalized human cell lines which

19 we love and honor like 293 and Hela, et cetera, et

20 cetera, have very short telomeres. These telomeres

21 can be maintained at a length of one to two KB. These

22 cells seems to be perfectly happy with that.

23 So I think it's more the maintenance of
24 the telomere length rather than the absolute length of
25 the telomere.

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1 If you artificially introduce telomerase
2 catalytic subunits into fibroblasts, what one
3 typically sees is that the best clones are ones that
4 build up telomere length to about 8 to 10, 12 KB and
5 then maintain it at that level. It seems to be a
6 function of the expression level of the H-TERP gene,
7 because if one does this experiment, you see cultures
8 that very slowly erode their telomeres. They will
9 eventually senesce.
10 You see cultures that build up telomeres
11 to maybe 20, 30 kilobase in length. That doesn't seem
12 to be good for them because the rate of growth goes
13 down. So really the best cultures are the ones that
14 maintain at least in fibroblasts. So I think it's the
15 maintenance rather than the absolute length.

16 DR. HUGHES: Would you please comment on
17 Carol Greider's knockout mice?

18 DR. SEDIVY: Well, yes. I didn't get into
19 that at all because that's at least for the time being
20 -- there are some paradoxes here. Okay? The obvious
21 paradox is that mice have extremely long telomeres, 60
22 KB on average. This is the laboratory mouse.
23 *Musculorattus* has perfectly normal telomeres, for
24 example.

25 In fact, this is really the other way

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1 around because these cells live for a very short time
2 in tissue culture. When they undergo senescence, they
3 undergo senescence with telomeres that are 50 KB in
4 size as opposed to 60 KB in size.

5 So there are really two answers to that.

6 One answer is that if you look at individual telomere
7 ends, you will see that there's a certain degree of
8 heterogeneity in that in fact in all cells, including

9 mouse cells, you always see a minority fraction of
10 chromosomes that have very short telomeres. If the
11 signal is caused by a perfectly short telomere that
12 gives, for example, DNA damage-like signal, then you
13 only need one per cell to give the senescence signal.
14 So that's one explanation. I don't really know
15 whether it's correct.

16 The other explanation is that mouse cells
17 don't senesce. They neither undergo crisis or they
18 undergo senescence. In fact, that plateau that we are
19 seeing during the immortalization is not senescence.
20 It's a differentiation-like process.

21 There are people, including myself, that
22 given this kind of loose distinction between
23 quiescence, differentiation, and senescence, would
24 prefer to define senescence now as a process that is
25 triggered by telomere erosion. Obviously when a mouse

1 primary fibroblast culture undergoes senescence, it's
2 not doing it, probably not doing it because it's

3 receiving a telomere signal.

4 I don't know if that is—does that make
5 sense?

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

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

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

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

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

22 DR. HUGHES: (Inaudible.)

23 DR. SEDIVY: Well, I think I just offered
24 you one explanation for that. That is that what we
25 are calling senescence is not senescence. It's

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1 something caused by some insufficiency in the median
2 that is in fact triggering a differentiation event.
3 Actually, Jim McDougall and I also don't quite agree
4 about what's happening in his keratinocyte cultures
5 because what he is calling senescence, some
6 keratinocyte biologists would prefer to call
7 differentiation.

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

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

16 Do these cells that enter a so-called

17 senescence have high levels of P21 or P16 or something
18 like that?

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

20 AUDIENCE MEMBER: So there is a signal
21 then.

22 DR. SEDIVY: If you take a knockout mouse
23 for P21 that doesn't undergo senescence. It just
24 keeps going. But you know, that's what I was trying
25 to say. That is that op regulation of P21 is not a

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1 molecular marker for senescence. P21 is op regular
2 because of oxidated stress, osmotic stress,
3 differentiation signals. This is a very general
4 machinery that is used to establish cell cycleresce.

5 I, in fact, don't know of any molecular
6 marker that is specific for senescence. This includes
7 the famous senescence-specific betagalactocytis
8 activity. You know, you see a lot of people staining
9 cells, and they turn blue and they say it's
10 senescence. Everybody knows if you put hydrogen

11 peroxide on your cells, they turn blue as well.

12 AUDIENCE MEMBER: Just a brief comment for

13 those people who might be setting up assays that would

14 be monitoring P21 sip. We, as I showed, found that

15 P21 is elevated in a subset of the HPB infected cells.

16 We did three other related assays. One was to look

17 for P21 MRNA. It turns out it's abundant in all

18 differentiated cells. But there is a post-

19 translational control on the accumulation of P21.

20 It turns out what happens is that if there

21 is not a signal that unscheduled in a synthesis is

22 underway, namely, abundant cyclin E, then proteosomes

23 rapidly degrade the P21 that's translated. When we

24 put in proteosome inhibitors, P21 piled up in all

25 cells and all replication was blocked.

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1 We went on to ask one additional question.

2 That is, how does P21 actually block S phase or DNA

3 synthesis. Unexpectedly, it had nothing to do with

4 blocking cyclin E activity. It turns out cyclin A,

5 CDK-2 or cyclin A CDC-2, can phosphorylate DNA

6 preliminary cell and all these other subunits I

7 showed.

8 The one thing cyclin A can't do is bind to

9 PCNA. But when the P21 sip piles up in these cells

10 that have excessive cyclin E, the way the P21 is

11 actually functioning is by binding to the PCNA and

12 blocking elongation, not initiation.

13 DR. LEWIS: This may be a naive question.

14 Is there any change in the activity of endogenous

15 oncogenes in cells at about the time they are entering

16 into senescence, especially rodent cells?

17 DR. SEDIVY: By activity, you mean level

18 of expression? I am not aware of that.

19 AUDIENCE MEMBER: What happens in

20 spragues? I mean are they different than

21 muskolorattus? Do they have shorter telomeres that

22 they go through?

23 DR. SEDIVY: What happens in spragues is

24 exactly the same that happens in muskolor.

25 AUDIENCE MEMBER: So even though one has

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1 60 KB and one has 2 KB?

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

12 DR. NABEL: John, I am going to just end
13 with one last question. You referred to the notion of
14 program of senescence and it being dominant. I am
15 just wondering, has anyone ever done a cell fusion
16 experiment where you have taken cells approaching
17 their limit and then fused to neo-natal cells. Is it
18 in fact dominant?

19 DR. SEDIVY: Yes. Those are very old and
20 classical experiments. In general of course,
21 senescence is a dominant state.

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

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1 substrates.

2 DR. FALLAUX: First of all, I would like
3 to thank your organization for inviting me here. The
4 subject of my talk will be on the generation and
5 characterization of new helper cell lines for the
6 construction, provocation, and protection of
7 recombinance replication effective adenoviral vectors.

8 In the past few years, the interest in
9 vectors derived from human viruses. This is caused by
10 the fact that from the many years of intensive
11 fundamental research on human adenoviruses, it has
12 been found that adenoviruses have several favorable

13 characteristics, including high stability of variance.
14 The variance is very easy to grow into pure with very
15 high fibers. It has a very broad host range.

16 Importantly, it has the capacity to transduce non
17 mitotic cells. This makes adenovirus a very potent
18 gene therapy.

19 It is known that it has very low kinisity,
20 and there is there ample experience with adenoviruses
21 as vaccines.

22 This slide shows a schematic
23 representation of the adenoviral genome. It is a
24 double stranded linear DNA molecular of approximately
25 36 KD, carrying several genes, flanked by inverted

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1 hermal repeats. The genes are sub-divided in so-
2 called early genes and in late genes, depending
3 whether they are stressed early or late during the
4 lytic infection.

5 This slide shows you a scheme of the
6 classical methods to construct the common

7 adenoviruses. All currently used adenoviruses carry
8 a deletion in E-1. This renders the virus replication
9 effective, and it also provides space to insert
10 therapeutic genes.

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

16 In addition, it also needs the
17 construction of an adaptor plasmid which carries the
18 transcriptase unit, including *geno-fenchfras*, but also
19 the left inverted herminal repeat and a part of the
20 adenovirus sequence which is also present in the large
21 fragment. Pro-construction of these two moleculars in
22 so-called helper cells, and the helper cell is the 293
23 cell made by Frank Reim. Upon close inspection,
24 another mination occurs, creating now the recombinant
25 adenovirus, carrying the gene of interest at the

1 position that we want.

2 You can propagate these elongated viruses
3 due to the fact that the helper cells complement the
4 missing elong function.

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

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

19 Another issue is the occurrence of
20 replication competent adenovirus, abbreviated RCA. I

21 will focus on this topic. There are various sources
22 of replication competent adenoviruses. In a sort of
23 infection, during the production of viral, or as an
24 earlier stage, or during the construction of the
25 recombinant factor, especially when you use classical

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1 methods, if you use the large clonon fragments. If
2 the digestion is not complete, you have RCA, namely
3 the wild virus.

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

10 Well, how does it work, homologous
11 recombination resulting in RCA? This is a scheme of
12 a typical elongated factor. This is a scheme of the
13 integrated adenovirus sequences in the helper cells.

14 The helper cell line is 293, and more

15 recently, we made alternative cell line 911. Both
16 helper cells carry the evon A, evon B in chorion
17 regions. But in addition, they also carry sequences
18 that enclose structural protein lines, downstream of
19 evon B, and upstream of evon A, be left for determinial
20 repeat.

21 Those sequences are also present in the
22 factors. So that a sequence overlap 5 prime and 3
23 prime of the therapeutic gene. As a result, you can
24 get homologous recombination by which the recominance
25 virus now trades its therapeutic gene for region E-1,

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1 and becomes replication competent. Now what you can
2 do about this is to avoid the sequence overlap. We'll
3 come back to that later.

4 Well there are only a few helper cell
5 lines available when you work with recombinant
6 adenoviruses. We are fortunately in our lab to have
7 three of them, including the two in our free cell
8 line, recently an iomosa line, and even more recently,

9 the PER cell line. All three cell lines are obviously
10 of human origin. They are all derived from primary
11 diploid embryonic cells. 293 is derived from kidney
12 cells, 911 from retinoblasts, and PER C-6 as well.

13 Now when I started to work with
14 recombinant adenoviruses in our lab, which is the lab
15 of Professor van der Eb at the Leiden University, I

16 used obviously 293 cells, and I met with some
17 technical difficulties. Since we had a panel of adeno
18 virus transformed human cells, including cells of
19 kidney, lung, and retinoblasts, I decided to screen a

20 panel of cells in order to find an alternative for
21 293.

22 From this panel of cells, I selected one

23 particular retinal cell line. We named it cell line
24 911. The reason for this name was to get the
25 attention of our colleagues in the U.S.

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1 (Laughter.)

2 Well, the construct we used to make the
3 911 cell line is shown over here. It carries the
4 adenovirus sera type 5 nucleotides 87 to 5,788,
5 including evon A and evon B.

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

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

20 However, I have shown you the construct we
21 used to generate the 911 cell line. We now have a
22 situation which is similar to 293. Namely, and also

23 in 911 cells, besides evon A and evon B encoding
24 sequences, also sequences of the left inverted termin
25 are repeat, and sequences in part encoding protein 9

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1 are present. So again, there is overlap and you can

2 create RCA.

3 So what we decided to do is start all over
4 again and make now the cell line in combination with
5 a so-called matched vector, now sequence overlap.

6 What we did was to make a so-called packaging
7 construct carrying only the evon A and evon B encoding
8 sequences in which evon A is driven by PGK, a

9 heterologous promoter, and a heterologous poly and
10 signal, and lay matched vectors that are deleted of
11 exactly that elong region which is present in the
12 packaging construct. Thus affording sequence overlap

13 and thus eliminating homologous recombination as a
14 source of RCA.

15 This shows you one of the packaging

16 constructs we constructed. Present are adenovirus
17 sera 5, sequences four, five line to 3,511. Those are
18 only the evon A and evon B encoding sequences. Evon
19 A is driven by the human PGK promoter. Evon B is
20 under its natural promoter, and directly flanking the
21 evon B stop codon as the polyadenylation signal
22 derived from hepatitis B virus.

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

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1 We did this because first we only had a few frozen
2 ampoules of the retinoblasts. Second, the packaging
3 construct contains several PCR fragments.

4 This slide shows you some of the functions
5 or features of adenovirus evon A. The features are
6 that domains 1 and 2 are involved in the regulation of
7 expression of genes. Evon A is known to associate at
8 the protein level with cellular proteins, P-105 RB,
9 cyclin A, P-300. I think the list is growing. All

10 these different features result in the transformation
11 and immortalization.

12 Not shown is the feature or function of
13 evon B. Evon B prevents the cells from growing into
14 apoptosis as a result of the activities of evon A.

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

23 This is caused by the fact that expression of evon A
24 in primary cells, in the absence of evon B, is toxic,
25 causing apyltosis.

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1 You can see this in the second line.

2 Transfected this plasmid to come in plasmid in evon B,
3 and we did obtain reasonable amounts of foci.

4 Obviously also the construct we used to
5 generate 911 resulted in focus formation.

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

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

15 Now we found that all clones expressed
16 very high levels of evon A in both 55 and 21 K evon B,
17 when compared to 293 and 911. We also looked at
18 vector use. We looked at three clones, clone 3, 5,
19 and 6. As you can see, we found that the three PER
20 clones tested exhibited similar use of recombinant
21 viruses, compared to 293 and 911. Since PER C6 played
22 the highest use, we decided to analyze this clone in
23 further detail.

24 Now of course the major issue for us was
25 to test whether or not our approach to use the PER

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1 cells in combination with matched adenovirus vectors
2 would reduce or even eliminate the generation of RCA.
3 This testing has been performed at the enzyme.
4 What I did was to amplify an RCA free
5 master stock of a typical adenovirus vector and
6 amplify it to 293 or PER C6. What I found, I can
7 summarize it for you, is that amplification of 293
8 resulted in RCA positive vector batches in
9 approximately 50 percent of the places.

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

16 So we concluded that our strategy to make
17 a PER cell in combination with new matched vectors

18 severely reduced, maybe even eliminated the RCA
19 problem, at least by homologous recombination.

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

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

5 We have a master cell bank available for
6 PER C6, also a working cell bank. Importantly, the
7 PER cells were made on a GLP conditions, using
8 certified U.S. bovine serum and trypsin. Currently,
9 InterGene is doing all kinds of tests which were
10 necessary for the use in the chemical setting,
11 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?

6 DR. FALLAUX: Actually we did not test
7 that yet. However, we did test this for the 911 cell
8 line, the weakly tumorigen in nude mice. So you might
9 expect the PER cells would exhibit the same feature
10 with respect to that.

11 DR. FRIED: What is weakly tumorigen?

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

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

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

25 Your second question. This may sound

1 silly, but we are currently trying to find out the
2 information of the donor. The cells were isolated in
3 the early 1980s, and we're now working backwards to
4 find out those details.

5 DR. NABEL: John?

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

14 DR. FALLAUX: In fact, all adenovirus
15 transformed cells are as highly transfectible as 293
16 cells.

17 DR. NABEL: Okay. If there are no further
18 questions, then I think we can just proceed onto the
19 panel discussion. John Coffin will chair that. If

20 the panel members want to come forward and get
21 started.

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

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1 seriously. So we probably should shoot for an hour in

2 which we either have a lot or a little to do,
3 depending on the will of the crowd and our host.

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

13 Tentatively we'll plan to sort of split

14 the discussion half and half between these issues, but

15 I think we can play that by ear as we go along.

16 Again, I expect widespread audience participation,

17 particularly since these are topics that I myself am

18 not really actively working in and am familiar with.

19 The questions that on the first part, on

20 the designer cell substrates, that we were charged to

21 address are summarized on this overhead. Before I

22 turn it on, I want to apologize in advance for two

23 things. One is my handwriting is very bad, so you are

24 going to be subjected to that for a while. Secondly,

25 I was given a rather blunt instrument to write with.

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1 Thirdly, of course I'm not well enough organized like

2 some of the previous chairs who have prepared these

3 ahead of time.

4 So this is a paraphrase, I hope an

5 accurate one, of the issues that were raised, that are

6 raised in the points to consider. The first regarding

7 designer cell substrates is the issue of whether cells

8 that are derived by the kinds of defined means that we

9 have seen, and we have seen the example of

10 introduction of telomerase plus or minus oncogenes,

11 viral oncogenes, or viral oncogenes alone whose

12 function among other things was to stimulate or

13 inactivate genes that are involved in senescence.

14 Whether cells that are created in this way

15 in fact offer significant safety, create safety issues

16 relative to other cell lines, whether they offer

17 advantages or disadvantages, whether we can go through

18 the sort of defined risk algorithm that was given to

19 us at the beginning of the meeting, to address these

20 and anything else.

21 So if we could get onto the first point

22 here. Are there significant safety issues relative to

23 tumor or neoplastic cell lines? In other words,

24 uncharacterized, what we should call it perhaps,

25 uncharacterized cell lines. Cell lines that have just

1 been handed to us either in tumors or that have arisen

2 by means we don't really understand very much about in
3 culture.

4 Would anybody on the panel care to --

5 DR. SEDIVY: Yes. I would just like to

6 make a very brief comment about designer cells. I was

7 asked to talk about the history of immortalization.

8 In fact, what my lab works on is more related to

9 interventions, genetic interventions in human cells.

10 So you know, obviously we have talked about putting an

11 H tert and putting in various viral oncogenes. So

12 really here the issue is can we make a cell line that

13 is immortal and it has a particular spectrum of

14 phenotypes that we want by absolutely defined genetic

15 interventions. I think the answer now is yes. We can

16 do a lot better than putting in SV-40 large T or E-1A

17 or E-7, because as have heard even today, we don't

18 really know exactly down to the last T what these

19 viral proteins are doing to the cell.

20 So in fact, what we can do is we can

21 delete cellular genes using gene knockouts, and

22 produce very much the same effect. That is, we can
23 really now contemplate really designing cells without
24 the use of viral oncogenes. I think that putting an
25 H tert is obviously a necessary step, but this is a

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1 cellular gene. So I think that's probably okay.

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

9 What's the position with telomerase immortalized?

10 I assume actually they are mimicking much
11 more the kinds of cell type that Dr. Hayflick would
12 define as—I forgot what he used, apologies—the
13 first stage of cell strains. That probably do not
14 have those particular phenotypic properties. Do you
15 know what the stage of those cells are?

16 DR. SEDIVY: Well, you know, we have a
17 really limited experience. This game has only been
18 played for a few months, maybe a year in some
19 privileged labs. I think what we're really talking
20 about here is proof of principle. In my lab, we're
21 not interested in growing cells in fermenters. We are
22 interested in cell cycle progression. But I think if
23 somebody wanted to make a cell line that grows well in
24 fermenters, I think it may be a good idea to
25 contemplate some of these new approaches.

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

9 be what you want.

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

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

24 If you don't want tert, you know, tert now
25 comes flanked with lox sites, so that you can take it

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1 out later on if you are interested in that.

2 DR. COFFIN: Can you be more specific

3 about what you would knock out?

4 DR. SEDIVY: Pardon me?

5 DR. COFFIN: Want you to be more specific

6 about what you are knocking out. Which genes have you

7 knocked out?

8 DR. SEDIVY: Well what we are interested

9 in doing is we are interested in dissecting the

10 machinery that establishes senescence. So not in a

11 single cell line, but at this point, we have P-21, cip

12 1, RB, B-53, and P-16 ink 4A knockouts in various

13 combinations. So you know, the vectors are available.

14 One interesting point about human cells

15 that is interesting to the technocrats I think is that

16 until this time, we do not see the requirement for

17 isogenic DNA. So in fact, these vectors that we have

18 made, and some other labs have contributed to this,

19 can be used and essentially—and have been used in

20 any human cell with equivalent frequency of

21 recombination.

22 DR. HAYFLICK: Yes. We have a lot of

23 information about the biological properties of H tert
24 transformed normal human cells now. There are the
25 ones with which Choma is familiar, are now as I

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1 indicated several days ago, approaching, and in one or
2 two cases beyond 400 PDLs. Many of them are beginning
3 to show some signs of aneuploidy. They are still
4 anchorage dependent. Their virus spectrum seems to be
5 identical to that with which we are familiar prior to
6 H tert transformation. Finally, the studies that have
7 been done on animal inoculation of these cells
8 indicates that they are not neoplastic. So that we do
9 know that much.

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

17 that that explanation defines about 25 years ago.
18 It's called SUSM-1. It's freely available. It is
19 from a normal human diploid fibroblast transformed by
20 multiple exposures to MMNG.

21 A second class of cells that also falls
22 into this category of normal human cell populations
23 transformed with something other than a virus, by way
24 of example, is KMSD-6 transformed by a former student
25 of mine, using multiple exposures to cobalt 60

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1 radiation. That cell population was also described
2 and in fact is used commercially today. It was
3 described about 25 years ago.

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

8 I would also like to repeat what I
9 mentioned the other day because it's been restated
10 again, and it's not accurate. That is, that there are

11 spontaneously transformed normal human cell
12 populations. I will be happy to provide the
13 references to whoever would like to have them.

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

19 DR. MINOR: From the point of view of the
20 infectious agent side of things, I mean I don't see
21 much difference between, you know, a brand new
22 tumorigenic cell line that appears in your hand and
23 one that's actually being designed to actually appear
24 like that. It seems to me they are both
25 uncharacterized and you would have to look at both of

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1 them very carefully. I'm not sure that you have new
2 infectious issues simply because you designed it to be
3 transformed.

4 DR. FRIED: Something came up the first

5 day. Would it be worth getting new cells that really
6 grow well with defined media so we can avoid serum and
7 any problems that come with that as something one
8 might think about?

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

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

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

24 DR. SEDIVY: You know, I am by no means

25 expert on this, so I can, you know, basically restate

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1 what I think is already out in the literature. That
2 is that you can definitely immortalize human
3 fibroblasts, pre-senescence fibroblasts by putting H
4 tert in. That's been shown now in a number of
5 laboratories.

6 You can also immortalize retinal pigmented
7 epithelial cells as reported by the Texas group. I am
8 not sure whether it's been published yet, but I have
9 heard that you can immortalize T cells, CD8 positive
10 peripheral lymphocytes.

11 Jim McDougall says that you can not
12 immortalize keratinocytes unless he said something
13 different yesterday, in that you need to interfere
14 with the RB pathway in addition to putting H tert.
15 There's also some indication that breast epithelial
16 cells may need an additional step to become
17 immortalized. That's all I know at this time.

18 DR. LEWIS: Since you can immortalize T

19 cells and you suspect that those would grow in
20 suspension like normal T cells, and you could grow
21 them in any large suspension culture, that you would
22 need to.

23 DR. SEDIVY: I don't think this work has
24 been published, so I think I'm just telling you
25 something that I heard at another meeting. So maybe

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1 we should just cool it. But I would presume that if
2 they grew in suspension before tert, they would grow
3 afterwards as well.

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

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

11 DR. COFFIN: But when doing that, of
12 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

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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 aneuploidy
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

7 --

8 DR. COFFIN: But you don't know what
9 happened. To bring a point to this, if one is
10 concerned about issues of what might happen with DNA
11 from the cells that was carried along, then it sounds
12 like, it sounds from what I'm hearing like there may
13 not be a great deal of difference between using these
14 cells and using these kinds of cells as compared to
15 using relatively uncharacterized cells.

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

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

24 DR. COFFIN: You can also use factors that
25 make these cell lines highly susceptible to viruses

1 you might want to be growing on them, and things like
2 that.

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

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

16 So any efforts toward trying to utilize an
17 episome are probably doomed to a degree of failure
18 because of the risk of—well, you have to maintain
19 episomal replication in addition to your chromosomal
20 replication. It just doesn't seem to work.

21 DR. COFFIN: And this of course, this kind
22 of issue gets far more amplified when we're talking
23 about growing up 10 to the 13th.

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

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

9 DR. ONIONS: Just as a general principle
10 about whether it's useful or not to engineer cells
11 rather than go out and select a transformed cell, a
12 pre-existing transformed cell from a tumor, it does
13 strike me that again, that it's under control and that
14 you have a number of choices.

15 The kinds of studies that PER C-6 has been
16 involved in give you a very precise engineered system
17 that's absolutely ideal for vector production. But it
18 perhaps also highlights with respect to the mistake
19 that was made. That is, that you had another
20 possibility here where you could actually choose the
21 cell. You could validate its origin. You could check
22 the person. That is the other advantage of you being
23 able to engineer materials, that you can actually pre-
24 select the actual source of the material that you
25 start with. That would have been an advantage that

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1 was unfortunately missed in this particular case.
2 That's not to undervalue the value of these cells, but
3 it does seem to me that that's what engineering cells
4 can give you. It gives you control at each stage of
5 the process.

6 DR. FALLAUX: Can I mention that it's
7 nowadays rather difficult to take primary human,
8 especially immuno material.

9 DR. ONIONS: It was—I understand only
10 too well. It's not at the end of the day a criticism
11 of PER C6 success, which I think are excellent. But
12 really just that where possible, that that should be
13 done.

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

20 The points we were asked to address also
21 included the use of the defined risk algorithm that
22 was mentioned at the beginning of the meeting, to
23 evaluate these kinds of things. Andy Lewis had that
24 on his slide, which I have asked him to put back on.

25 This will also, I mean with this, we will

1 sort of segway into the general discussion as well I

2 think, because these are the issues.

3 So the question is, can we go through and
4 do this, and is it possible in this particular case,
5 just using this as an example of this kind of
6 procedure, to assess the level of risk posed by these
7 issues, infectivity, infectious and so on,
8 quantitatively. My own feeling right now is that
9 we're no where near a position to do this, certainly
10 for DNA issues. We might be able to put some sort of
11 numbers on viral issues. It's a little hard to see
12 exactly how because there's so many different ones,
13 which could have a different contribution. But maybe
14 we can get some further comments on these sort of
15 issues from the panel.

16 DR. ONIONS: My only comment, and I
17 understand why a defined risk approach was used, and
18 it certainly makes you think. I mean that's one of
19 its great virtues. I think one of the real intrinsic
20 problems, that if you applied, you can give yourself
21 a false sense of security. It would strike me that
22 Phil's story about SV40, if it turns out that is the

23 origin, SV40 in people, would have given you such a
24 false sense of security, I think, because you might
25 have come through that exercise in the 1960s. I'm not

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1 sure how you would have predicted that that agent was
2 there a priori.

3 So I'm not sure that you can give
4 guarantees that 1 and 10 to the 6th dose is one-half
5 X, if you don't know what X you are looking for.

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

10 (Laughter.)

11 Because I think one thing it will do is it
12 will tell you where you think you are confident, on
13 what stage of the process you are actually confident.
14 Then you can actually question whether your confidence
15 is misplaced or not. But I think if you come out with

16 a number, I think you are asking for trouble.

17 DR. LEWIS: Yes. I don't think that we
18 discounted that. I think in sort of going through
19 this thing, what we were trying to do was to figure
20 out exactly where we can be reasonably confident of
21 what we're doing and where we can not be confident of
22 what we're doing. But we always recognize that when
23 push comes down to shove, the bottom line is that
24 we'll always—we can never be sure.

25 So at some point in time, it requires a

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1 leap of faith to say this product or this cell line,
2 or whatever, can in fact be used. I think what we're
3 trying to figure out a way to do is to make sure that
4 when we get to the point or we have to make that leap
5 of faith, that it's better to find than it would be if
6 we did nothing at all.

7 So the attempt here is to develop sort of
8 a way of thinking about narrowing that margin of
9 error, or at least to develop a margin of error that

10 is better than it would be if we're just doing it on
11 intuition.

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

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

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1 with which I'm fundamentally uncomfortable, if I had
2 a better notion of how uncertain people were about the
3 assumptions they were making in generating the numbers

4 in the first place.

5 DR. ONIONS: I think that's what we're all

6 saying, is I think I started it off by criticizing the

7 whole approach. I think what Phil said is what I

8 think. I think Dr. Lewis said the same thing. That

9 is, don't believe the numbers. All it is is gives you

10 a manner of approaching what are the issues, really.

11 I think that's the way it should be treated.

12 I agree. I don't think anyone should

13 believe the numbers at the end of the day.

14 DR. HUGHES: I think it might help if when

15 someone put down a number, they at least put down a

16 range of numbers, and that would generate a range of

17 confidence at the end.

18 I think what people will see when they do

19 that, is that the ability to define the confidence

20 interval is going to expand when you multiply the

21 numbers together. I think that act may in a sense

22 help define how uncertain the number actually is.

23 DR. COFFIN: I think from a regulatory

24 standpoint, what often happens is that the far end of
25 the confidence interval on the worst possible side is

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1 taken, and then that's propagated through. You never
2 see the other side.

3 DR. ONIONS: I don't want to just go into
4 an academic discussion about risk assessment because
5 I'm not really interested in it, in that formal sense,
6 but there are two other approaches that are used. The
7 engineering industry uses a form of analysis that
8 doesn't do risk assessment like that. It actually
9 looks for holes. It looks for what could go wrong.
10 In a sense, that's really probably what we ought to be
11 doing. Then there are four mechanisms of that kind of
12 analysis.

13 The other form of analysis is the one that
14 has become fashionable in the U.K., which is this
15 concept of the precautionary principle, which
16 ultimately, it seems to me, means you don't ever do
17 anything because you never know what might happen,

18 which seems to me completely dumb.

19 DR. COFFIN: So we're voting against the
20 precautionary principle. You can't be sure of
21 anything, but you can be sure of that.

22 Are there any other points anybody would
23 like to make about this? One could say that this is
24 a useful way to organize your thoughts on this
25 subject, but shouldn't be taken as giving you either

233

1 additional grounds or comfort or discouragement,
2 unless you actually had a situation where you had
3 measurable quantities.

4 Are there any other issues or questions
5 regarding the designer cell substrate issue that
6 anybody wishes to raise?

7 DR. SHEETS: Hi. Becky Sheets, FDA. I am
8 going to ask the whole panel what I tried to ask Dr.
9 Hughes earlier. That is, the kinds of questions that
10 sponsors ask us. One question I would ask is we've
11 heard a lot of people in this meeting say that the

12 oncogenic DNA issue has been put to bed. Has it?

13 DR. COFFIN: That segways us into the
14 next, into the general discussion, which is fine.

15 Before we go into that, can we see if
16 there are any other specific issues regarding cell
17 substrates?

18 DR. SHEETS: Any questions about the
19 quantitative?

20 DR. COFFIN: If we do that, we can turn
21 off that slide, put up my next one, and then you can
22 ask the question.

23 DR. SHEETS: There's one question.

24 AUDIENCE MEMBER: Jerry Sato from Merck.
25 I understand the reluctance to put a number on

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1 something when you have such degree of lack of
2 confidence and the assumptions that are going into it.
3 But I do think that getting an order of magnitude of
4 where we are is actually helpful in our thinking about
5 what we feel comfortable going forward with or not.

6 When you have a lack of confidence in each
7 of those areas, you also have to ask the question,
8 what are the chances that all of your assumptions are
9 wrong? In other words, are two of them wrong, three
10 of them wrong, five of them wrong, seven of them
11 wrong? Because you have to put a degree of that's not
12 likely to happen. So if you multiply the lower end of
13 the confidence interval for all those things, then you
14 will never do anything. But that's not the way it
15 works in reality.

16 So I think it would be useful for somebody
17 from the engineering community, where they design
18 bridges that aren't supposed to fall down and other
19 things, to try and put a bit little more
20 sophistication into this analysis, because in the end,
21 somebody is going to ask our community, which is the
22 regulatory community, the academic community, and the
23 industrial community, for the number or at least what
24 they thought the number was when they went forward
25 with their act of faith. Because there is a certain

1 amount of common sense that goes into it, which is the
2 basis of the act of faith. Then there is whatever
3 kind of quantitation we can put into it. It's the
4 combination of those two things that I think we are
5 going to have to reassure the general public about.

6 DR. HUGHES: I would recommend to you the
7 book *Strategy and Conscience* by Anatol Rappaport,
8 which attempts to deal with the issues having to do
9 with what was called strategic thinking, in which you
10 calculate, for example, the probability of some
11 unlikely event, such as thermonuclear war. Mr.
12 Rappaport does a very good job of making clear why
13 doing the calculations when you don't have the proper
14 data is in fact a very risky and misleading
15 proposition.

16 AUDIENCE MEMBER: I guess it might be
17 worth pointing out that there are, however, some cases
18 where you clearly do have the proper data. Right?
19 You know the sensitivity of a specific assay for a

20 specific adventitious agent, if in fact you choose to

21 figure out what it is.

22 So you can actually answer, based on those

23 kinds of questions, and based on that kind of data,

24 the specific question of how sure are you that

25 something isn't there. If a better assay comes along,

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1 and it's still negative, then you can say by how much

2 more certain you are.

3 So just because you can not come up with

4 good estimates for some of the numbers, seems to me it

5 would be crazy to throw the baby out with the bath

6 water and claim then that you shouldn't attempt to

7 come up with good numbers for those things that you

8 can.

9 DR. RABINOVICH: Gina Rabinovich, NIH. A

10 non-regulator asking a question from experience

11 learned this summer, in which we have been dealing

12 with using a quantitative number, i.e. the numbers

13 that the Federal agencies and the global agencies has

14 set for acceptable limits of mercury, i.e. methyl
15 mercury, and then trying to attempt to understand what
16 those uncertainty factors mean for thimerosal and
17 vaccine exposure im.

18 The concern I have, and I think it has
19 been heard, is that these numbers take on a life of
20 their own. They become the standard against which
21 things are measured. So that that kind of concern
22 needs to be entered into, attempting to use the data
23 when those data do exist, but understanding the limits
24 to it.

25 DR. COFFIN: That's inherent in

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1 regulation, that things become standard.

2 DR. LEWIS: Lewis, FDA. To follow up a
3 little bit on what Phil was saying. I think one of
4 the areas that we could approach with some confidence
5 is the ones who saw Keith Peden's data last night
6 using the tac man assay to detect JC, BK, and SV50 in
7 human tissues.

8 Now if someone comes in with substrates

9 they derive from a neuroblastoma, which we learned at

10 the DNA tumor virus meeting a year or so ago, is it's

11 usually contaminated with BK virus. We wanted to be

12 sure that there was no BK virus in that substrate.

13 Then we could apply this assay with a fairly

14 sufficient level of sophistication and say with some

15 certainty, based on the volumes and things that were

16 tested, the level at which that particular genome or

17 that particular virus is absent. So I think that we

18 sort of view that as a possible starting point for a

19 quantitative approach.

20 Now obviously you can't do that unless you

21 know exactly the probes and things that you are

22 working with, and you define the limits of their

23 ability to detect things. But I think this is one of

24 the sort of examples that was going through our mind

25 when we were thinking about how to do this.

1 So you start at the place where you might

2 be able to generate some relevant data that's
3 meaningful. The other stuff will fall into place as
4 we get better.

5 DR. ONIONS: I mean I think that's exactly
6 right. I think more and more that we move to assays,
7 that we get good quantification on, we can validate
8 them, and it's the sensitivity and limits of
9 detection. I think all of that is absolutely
10 essential. I mean I absolutely 100 percent concur
11 with that. I think it does add confidence to those
12 specific questions.

13 I think when you are asking specific
14 questions, then I 100 percent agree. I think my
15 concern is perhaps that where you try and make
16 assumptions, for instance, of residual DNA. I mean I
17 was the one who said I thought it should be put to
18 bed. That is because all the evidence that I had
19 heard didn't convince me that there was a risk.

20 On the other hand, I actually believe that
21 no one has done the right experiment to actually

22 convince me of that, in a formal scientific sense. So
23 we're then dealing with the area of conjecture. That
24 conjecture is based on non-quantitative data.

25 DR. COFFIN: We're leading into you,

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1 Becky.

2 DR. SHEETS: I'm patient.

3 DR. COOK: I'm sorry. Jim Cook. As I was
4 sitting there thinking about how you would describe
5 these issues to a patient or to a group who is asking
6 you about the wisdom of using a vaccine, it seems like
7 in addition to trying to generate some logic about
8 calculations of numbers and risks, that every
9 opportunity that you have, it would be worthwhile
10 going back to history and saying well, we have done
11 virtually something like this along the way ever since
12 vaccines have been developed, and the experience with
13 this approach has been the following.

14 So maybe there could be some real numbers
15 in a historical sense, used to color or give some more

16 real meaning to these, what are otherwise theoretical
17 things, to help communicate this to the public, as
18 well as to provide some, a little bit more logic than
19 just phenomenology to the calculations that are being
20 made now. So if history is used to color the
21 estimations, that might be of some use.

22 DR. COFFIN: How comforting is it to tell

23 --

24 DR. COOK: Say it again?

25 DR. COFFIN: How comforting is it to tell

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1 patients that there are three or four cases of

2 paralytic polio?

3 DR. COOK: Well, I think you have to be

4 honest with them. You say look, you are one of 250

5 million people. The odds for your child is the

6 following, and I think it's a very good idea to use

7 this vaccine.

8 It is those kinds of conversations that

9 eventually lead adolescents into getting hepatitis B
10 vaccine. We're doing a miserable job of this, by the
11 way. I think as a culture, you know, if we bat 50
12 percent, we're lucky.

13 DR. COFFIN: That's clear in light of
14 things in the movies, because lately we're doing a
15 terrible job where these people show up with these
16 anecdotal cases of somebody's child gets vaccinated,
17 and then two months later is diagnosed with autism.
18 It's automatically due to the vaccine.

19 DR. ONIONS: But I think history can also
20 be a dangerous thing. I mean the British government
21 has been criticized, partly justifiably, but I think
22 partly unfairly for the problems of the way BSE
23 problem goes up.

24 But the issues concerning public health
25 were based on people were asked what is the risk, what

1 is the risk of the human population of the BSE
2 outbreak, when we had a few hundred cases of BSE in

3 cattle. Well, there were only a few hundred cases.
4 The general assumption was, and it was a widely shared
5 general assumption by those who were informing the
6 area, the people who worked on scrapie and so on,
7 well, scrapie has no evidence whatsoever of scrapie
8 transmission to man. We have been eating scrapie-
9 infected sheep for generations and it doesn't seem to
10 have been transmitted to people. There is no evidence
11 of that whatsoever.

12 The probable likelihood that BSE is of
13 scrapie origin that's gone through the rendering
14 process, because we have never seen a spontaneous
15 spongiform encephalopathy in cattle, so that's
16 probable origin ipso facto, you know, there's no
17 problem.

18 It wasn't quite as glib as that because
19 actually within a year, all the controls on human food
20 were in place and so on and so forth. They were badly
21 conducted, but they were theoretically in place.

22 So I think history can also be dangerous.

23 I'm not sure that we can always learn the right
24 lesson.

25 AUDIENCE MEMBER: I get your point, but I

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1 think there is a difference between an ongoing
2 epidemic that's yet resolved, and the experience of X
3 number of hundreds of millions of doses of polio
4 vaccine.

5 So what I am saying is, if you are going
6 to make a calculation about the likelihood of one in
7 a million or one in 10 or 100 million happening with
8 a vero cell or with a primary cell, you can say well,
9 we've got experience making polio vaccine in vero
10 cells, and there have been the following hundreds of
11 million doses given, and the likelihood is that when
12 the incidence of the real disease gets so low that you
13 are finally going to see some background. That's the
14 real --

15 DR. ONIONS: I'm sorry. You
16 misunderstand. What you are saying, I absolutely

17 agree.

18 DR. COFFIN: Do you want to continue this
19 discussion or do you want to break into --

20 AUDIENCE MEMBER: Very similar aspect,
21 although a little bit less scientific. As I perceive
22 the discussion, of course there's this highly
23 sophisticated, highly conscientious scientific
24 community, and there's the general public on the other
25 side.

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1 The general public, to my perception,
2 consists of at least two sub-groups. One group that
3 is sort of generally benevolent and would believe
4 scientists. But there is a very strong group that is
5 not believing scientists. They are sort of using that
6 as a political tool to attract attention. In our
7 country, we have had this experience with the Green
8 Party, that has become very influential in the
9 European Union and maybe in other countries as well.
10 Now one thing I also, since we're ending

11 and coming to the end of this meeting, would like to
12 raise, isn't it the responsibility of scientists also
13 to do something to better educate the public? I know
14 this is a utopian goal, but at least if we could
15 increase enough of people in the general public who
16 are educated or better educated in science and biology
17 and biomedic issues, we would at least have a
18 political community that might support scientific
19 issues more valuable than we have had it so far.

20 You know, in our country at least, every
21 time when the Greens demanded to stop all biomedical,
22 all gene technology research, and it course never
23 happened. After they come to power, maybe they have
24 different outlooks on life. But I think as a
25 scientific community, unless we do something at least

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1 for the future, we might be in a very difficult
2 situation to defend certain issues.

3 If I confronted some of the violent
4 ideologically pure Greens in our country, because the

5 trick is, we have been discussing here, I'm sure they
6 would say "Shut it down because this is unsafe,
7 totally unsafe."

8 So what I am trying to recommend is we
9 have to do something to have more people in the
10 general public who can appraise and can assess the
11 difficulty and the uncertainty in any biological
12 research. We can never get down this figure to 10 to
13 the minus 80. So we have to raise understanding on
14 the other side.

15 DR. SHEETS: So have we put oncogenic DNA
16 to bed?

17 DR. COFFIN: No. I would like to use the
18 sort of summary—I think that's in a sense, an
19 overhanging issue. We have talked about infectious
20 risks and measurements and so on considerably today
21 and in the past few days. I think an overhanging
22 issue is this oncogenic DNA issue regarding the
23 specific charge of the meeting, which is the use of
24 tumor versus other kinds of cells, tumor and

25 neoplastic whatever, transformed cells versus other

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1 kinds of cells as substrates for vaccine production.

2 Although I think many of us here, perhaps
3 all that's here, feel this is not a risk to be really
4 concerned about in a scientific sense, I think many of
5 us here might agree that the issue is not completely
6 put to bed in the sense that we can't put any real
7 good numbers on it.

8 So now if you ask your question.

9 DR. SHEETS: Okay. Has oncogenic DNA been
10 put to bed?

11 DR. HUGHES: I will answer in two
12 different ways. I will give you my opinion, personal
13 opinion, and then I will tell you what I think should
14 be done, which is slightly different.

15 Personally, and this would apply if you
16 approached me to do something to myself, I am not
17 concerned. However, it is my view that the data that
18 we have, particularly for the consequences of putting

19 DNA into animals, is not sufficient to satisfy me as
20 a scientist. I am going to try to help my colleague,
21 Dr. Coffin, and some of my colleagues at the FDA to
22 try and organize a simple study that would be more
23 satisfying to me.
24 I think I would feel more comfortable if
25 we had more data that was of the experiments done on

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1 a larger scale under more controlled conditions. I
2 think that would give me a greater degree of comfort.
3 DR. ONIONS: I think I almost entirely
4 concur with that comment actually. I did say a
5 comment, and said it partly to be provocative, but on
6 the other hand, I think I share that opinion.

7 I have seen nothing that would convince me
8 at the moment on the data, which there's a singular
9 lack of, or just from I suppose theoretical reasoning,
10 to suggest that this would really be a significant
11 danger. But on the other hand, there are the tools
12 now, and I say yet again, but I think some of the

13 transgenic models offer that possibility for testing,
14 whether or not DNA is a risk. It can be done in a
15 series of graded experiments from taking the worst
16 case examples of actually simply just repeating—of
17 injecting oncogenes at various titrations into animals
18 that are already primed with oncogenes as a
19 transgenic, down to taking tumor DNA down to taking
20 normal DNA.

21 I mean those experiments, they are pretty
22 straight forward to do. I mean the interpretation
23 might be a bit more complex, but they can be done. I
24 think they are worth doing. It might help you to put
25 some limits, broad limits on the thing we're all just

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1 making conjectures about.

2 DR. MINOR: I don't think it's been put to
3 bed either.

4 DR. COFFIN: For the same general reason?

5 DR. MINOR: Broadly speaking. I mean I

6 think it's clearly a very, very complex issue about

7 how you actually induce a tumor. So you do your 3T3
8 assays and you pick up H ras. Okay? I mean it's an
9 artifact of 3T3s or was that just a question of how
10 common H ras is.

11 If you go and put your DNA in
12 intravenously, is that the same as putting it in
13 subcutaneously, for example? I mean if you put it in
14 because it's been picked up by an envelope virus, is
15 that going to make any—will it be picked up by an
16 envelope virus? Will that make any difference?

17 I mean it seems to me that there are so
18 many sort of loose ends to it that I don't think while
19 there is no evidence that DNA is tumorigenic, and I
20 buy that 100 percent, it doesn't seem to me that it's
21 necessarily been dealt with properly. That is why I
22 guess I am agreeing with what the previous two
23 speakers said.

24 DR. LOEWER: So as I already have said, I
25 personally believe that there's not a real big risk

1 with purely oncogenated DNA. Purely oncogenated means
2 three or five or six. But I realized that there is
3 still, I believe, lack of experimental data. This was
4 already mentioned by John Coffin, in saying that since
5 18 years, this question is on the table. Since 18
6 years, no additional experiments have been performed.

7 I would like to propose that regulatory
8 authorities, which are involved in regulation of these
9 biologicals, the major ones, that we should sit
10 together and to join the efforts and maybe decide on
11 experiments which can be done in the foreseeable
12 timeframe.

13 But I look forward to see what types of
14 experiments John may recommend.

15 DR. FRIED: I think most of the evidence
16 we have so far, which is limited, says at least
17 putting DNA into animals, we haven't seen anything
18 happen.

19 We have only seen in NIH3T3 cells, and we
20 now know that there is a defense mechanism in the cell

21 when it sees an oncogene. That is this P, this arf,
22 which is the alternative reading frame of P-16. So
23 it's like an immune system of the cell, specifically
24 for oncogenes. The radiation activation of P-53 is a
25 different pathway. This turns on, and arf activates

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1 P-53, and P-53 then closes itself to go through

2 apoptosis.

3 The only positive things are in NIH3T3
4 cells. They are the classic cell where the arf gene
5 is inactivated. Probably that's why people have been
6 using that for years. They are very easy to transfect
7 because maybe even transfection kills the cells in
8 terms of P-53.

9 But that said, I would like to see a lot
10 more injection of DNA from different tumor lines into
11 animals, and to really put it to bed.

12 DR. COFFIN: It's striking to me that one
13 of the very few, if only successful experiments with
14 injected activated oncogene DNA in chickens is the one

15 that Hsing-Jien Kung did that was off-site, or in any
16 animal, that was Hsing-Jien Kung. It was actually
17 fairly efficient sort of transient transformation of
18 cells, but transformation of cells is always
19 transient. It's virtually impossible to immortalize
20 them. They are much harder than human cells to
21 immortalize. It's been done once, to my knowledge.

22 So there may be something very
23 fundamentally different going on in that model,
24 because there may be some fundamental difference in
25 chickens as compared to mice, as compared to humans,

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1 which already have important --

2 DR. EGAN: Bill Egan from FDA. I would
3 certainly like to work with you and other regulatory
4 authorities to try and design and our colleagues in
5 PHS, to try and design these experiments and do these
6 experiments, and get the data, and get away from the
7 remark I quoted from Maurice Hilleman from 30 some-odd
8 years ago about this debate being a philosophical or

9 ecclesiastic debate because we simply don't have the
10 data. Here we are 30 some-odd years later, you know,
11 with the same question, with the same debate. It's
12 still opinion.

13 I must say I also feel that personally,
14 myself, I don't think there is a large risk from the
15 DNA. But then again, the kinds of risks that we're
16 talking about are very small risks, very, very rare
17 events. Things like one in a million are not
18 acceptable or in many cases are not acceptable. Those
19 are hard data to get.

20 While I may not feel there is a risk to
21 me, the bottom line basically for the approval of
22 almost any of these vaccines, is would I put this into
23 my children. There it becomes a much more
24 conservative process. If putting it into my children
25 is putting it into other people's children, it's the

1 same thing.

2 DR. HUGHES: If you want one more piece of
3 data that should give you some comfort, it is the
4 experience attempting to make antibody to the oncogene
5 sarc, which involved putting an avian virus into a
6 number of mammalian species under circumstances in
7 which the virus absolutely does not replicate.

8 In adult animals, to my knowledge, no cell
9 growth was ever seen. The only experiments that
10 succeeded, to my knowledge, were those initiated by
11 John Burge, in which he put enormous amounts of ras
12 sarcoma virus of a subgroup that would infect
13 mammalian cells. We are talking sort of 10 to the
14 10th infectious units. Into immunologically naive
15 baby bunnies. In those animals very transiently,
16 there were small nodules which regressed.

17 In those animals, you did see antibody to
18 the oncogene sarc, implying that there was transient
19 uptake of the DNA, at least probably permanent uptake
20 of the DNA. But even under those circumstances in
21 which the delivery of the DNA is extremely efficient
22 and every copy of the DNA carries a known potent viral

23 oncogene under circumstances where the DNA will not
24 replicate but will insert, do you see any permanent
25 transformation? Again, I'm not suggesting that this

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1 is sufficient. I am one of the advocates of more
2 experimentation. But the data that we have suggests
3 that this is not a simple process.

4 DR. EGAN: No, but I mean these are the
5 kind of data that start to put brackets around the
6 numbers for the levels of risk.

7 DR. COFFIN: Of course you have enormous
8 problems, including the fact that sarc is never seen.

9 It's a human oncogene. For many years, the most
10 popular viral model.

11 AUDIENCE MEMBER: I had a question. If
12 given the unknowns, and given the data that was
13 presented about hit and run DNA modification
14 potentials, would the panel in the context of this
15 type of vaccine development, and given the unknowns,

16 give the vaccine to someone with a strong family
17 history of malignancy or who was a cancer survivor who
18 we know is at increased risk for a second cancer, if
19 that was you or your family member?

20 DR. COFFIN: The question, to sort of
21 focus that a little bit, the question is whether we
22 would consider there to be a greater risk in certain
23 sub-populations who might have sort of pre-activated
24 oncogenes or some other fact of predisposing.

25 DR. ONIONS: I understand the question,

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1 but it sort of arose -- I will go backwards, because
2 one of the things that used to concern me was when
3 people were doing clinical trials with rusvel vectors,
4 and they were using marker studies. That sort of
5 study did worry me, because actually what you were
6 there doing was putting something, inserting something
7 into somebody who probably already had a preexisting
8 oncogenic hit. That struck me as being dubious,
9 mildly.

10 In this situation, I would have thought
11 that unless you have got somebody with a Li-Fraumeni
12 syndrome or something, that you are dealing with
13 changes that are somatic changes in a few cells, even
14 if they are going to risk of a second cancer.

15 So the likelihood that you hit the right
16 cell is pretty low. So I wouldn't have thought it up
17 the risk—I'm sure the risk has increased, but I
18 wouldn't have thought that risk has increased
19 significantly, unless of that sort where you've got
20 mutation.

21 DR. MINOR: It would also depend on what
22 you are trying to protect them against too, wouldn't
23 it?

24 DR. SHEETS: Before we lose our entire
25 audience, I wanted to—I think we have gotten a

1 pretty clear answer on the oncogenic DNA issue. I
2 wanted to ask another kind of question that FDA has
3 asked, before we lose our entire panel. That is, are

4 there risks, additional risks that one perceives in
5 using a continuous cell line such as vero cells,
6 particularly vero cells, which is immortal but not
7 tumorigenic at the levels that vaccines are made, past
8 the level that vaccines are made. Is that worse in
9 any way than diploid cells, for a live viral vaccine?

10 DR. MINOR: This is apart from the DNA
11 issue?

12 DR. SHEETS: Well, in a continuous cell
13 line, certainly there are—it may be aneuploid, but
14 it's not tumorigenic in animals. So you can comment
15 if you'd like about whether you think the DNA is
16 oncogenic.

17 DR. MINOR: I would say that the DNA from
18 vero is as questionable as the DNA from anything else.
19 I mean John, with whom we discussed these matters,
20 more or less said the same thing. I think that it
21 doesn't matter how malignant it is. Maybe it depends
22 on how many oncogenes you put in there. So I would
23 have thought that a vero is as questionable as

24 anything else, or is not as questionable as anything
25 else.

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1 DR. SHEETS: So you wouldn't suggest to

2 make a live viral vaccine in vero cells?

3 DR. MINOR: I think it would depend on the

4 live viral vaccine. I mean I think OPV clearly has

5 been made in vero cells. You can scrub it clean. I

6 think you can more or less destroy anything that's

7 actually hanging on the end.

8 I have more serious thoughts perhaps about

9 things like a paramixovirus vaccine, because you

10 couldn't clean it up so much perhaps.

11 DR. SHEETS: What about the sort of crude,

12 less purified live viral vaccines, not the purified

13 vaccines like we heard about last night with OPV, but

14 the things that are just filtered cell culture

15 supernatant?

16 DR. MINOR: Right. They might be fit.

17 I'll tell you that. Which is not to say there's any

18 good reason for me to be uncomfortable with them.

19 It's just that they make me feel uncomfortable.

20 DR. COFFIN: We are very fast losing our

21 audience, so I think --

22 DR. HUGHES: Isn't it partly the question

23 do you know the life history of your cells as opposed

24 to the state of the cells at the end?

25 DR. SHEETS: Vero cells are a bank that is

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1 well characterized. The reason for the question is

2 that we have numerous live viral vaccines of the sort

3 I described that are being proposed to be made in vero

4 cells. Manufacturers prefer vero cells because one,

5 they can be characterized. Two, you get a high yield.

6 Three, they can be grown in the sorts of fermenter

7 culture that you heard about.

8 DR. HUGHES: I'm not particularly bothered

9 as long as I know that the sort of life history of the

10 cell. But I think the question is, if you have a cell

11 that's been in culture for a long time and has had a

12 complicated culture history, do you know that history?

13 AUDIENCE MEMBER: I asked the last panel

14 the same question. It comes down to the question

15 really is the adventitious agent issue put to bed as

16 well. Do we now have the assays in place that can

17 easily be applied to validate the freedom from

18 adventitious agents of these kinds of new cell lines?

19 The answer that Dr. Broker gave in the

20 last panel suggested that one could attempt to use DNA

21 chips and things like that, which to my knowledge

22 aren't assays that at least tomorrow I could go out

23 and do on a cell line and give me some confidence.

24 So my question to you is, sort of using

25 the standard assays that you are all aware of, without

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1 developing further assays for this specific purpose,

2 do we have enough information to be sure that these

3 kinds of new cell lines are safe from the adventitious

4 agent perspective?

5 DR. ONIONS: That's another unanswerable

6 question, isn't it. I would just make the point that
7 I think you have to adopt somewhere between good
8 science and pragmatism. I mean you could
9 theoretically go and do representation difference
10 analysis on all these cell lines. Actually, I don't
11 think it's possible because you don't usually have the
12 partner. But theoretically you could do that. That's
13 not really a practical solution.

14 It does seem to me that we do know virus
15 types that tend to be latent in cells, and that it's
16 sensible to perhaps think of strategies of widening
17 the brief of detecting those agents, because I'm not
18 convinced that the kind of routine types of infecting
19 -- infectability assays when they work are as
20 sensitive as PCR, as just Phil pointed out. But I am
21 not convinced that always the right infectability
22 assay is present to actually detect certain agents.

23 So that you are probably relying on a combination of
24 things. Perhaps we do need to look at redundant PCR
25 for certain agents.

1 DR. COFFIN: I would think the producers
2 would have a big attraction, is set up the same assay
3 and use it for everything.

4 DR. MINOR: I mean I think you could also
5 argue that you have used these assays for looking at
6 human diploid cells and primary cultures, and all that
7 sort of stuff. Right? What's the difference in
8 principle in terms of adventitious agent contamination
9 between those and the cells you are looking at here?
10 I am not sure there's much difference. But are the
11 concerns as big or as little.

12 AUDIENCE MEMBER: For one, I would like to
13 thank Dr. Andy Lewis for bringing us together. This
14 has been a very stimulating week. Last night Dr. Vyas
15 showed us a picture of the thinker. But that actually
16 prompted me to remember that Rodin placed that
17 gentleman directly above the gates of hell.

18 (Laughter.)

19 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

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1 sponsors and those of us at CBER who worked on this,
2 we really appreciate the effort that the session
3 chairs, the panel chairs, and the speakers have put
4 into this meeting. When you attempt to put something
5 like this together, there's always a question of how
6 it's going to turn out. I think the success that we
7 have enjoyed here the past three days is a tribute to
8 the work, an incredible amount of work, that has gone
9 on on a very short period of time.

10 I think that I was very concerned when we
11 were trying to contact folks in May to do this by
12 September. For those of you who rose to the
13 challenge, I can't thank you enough on behalf of the

14 sponsors.

15 With that in mind, I hope everybody has a

16 great trip home. Get your papers in whenever you can.

17 Thank you.

18 (Whereupon, at 2:57 p.m., the proceedings

19 were concluded.)