FOOD AND DRUG ADMINISTRATION

AND

NATIONAL CANCER INSTITUTE

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WORKSHOP ON

TUMOR VACCINES

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Friday, December 11, 1998

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The workshop commenced at 8:00 a.m., in the Masur Auditorium, Building 10, National Institutes of Heath, Bethesda, Maryland.

SPEAKERS PRESENT:

RAJ PURI, M.D., Ph.D.

PHILIP D. NOGUCHI, M.D.

BERNARD A. FOX, Ph.D.

GREGORY PLAUTZ, M.D.

ERNEST W. YANKEE, Ph.D.

JAMES J. MULE, Ph.D.

DONALD L. MORTON, M.D.

RICHARD A. YOUNG, Ph.D.

SPEAKERS PRESENT (Continued):

MICHAEL G. HANNA, JR., Ph.D.

JEANNE M. NOVAK, Ph.D.

MARVIN I. SIEGEL, Ph.D.

JAY P. SIEGEL, M.D.

MARIO SZNOL, M.D.

STEVEN A. ROSENBERG, M.D.

JEFFREY S. WEBER, M.D.

PETER P. LEE, M.D.

H. KIM LYERLY, M.D.

MARY L. (NORA) DISIS, M.D.

JEFFREY SCHLOM, Ph.D.

JAY BERZOFSKY, M.D.

NICHOLAS P. RESTIFO, M.D.

PATRICIA KEEGAN, M.D.

SAMIR KHLIEF, M.D.

RICHARD M. SIMON, D.Sc.

JONATHAN W. UHR, M.D.

JAY J. GREENBLATT, Ph.D.

DAVE S.B. HOON, Ph.D.

CARLETON C. STEWART, Ph.D.

ABDUR RAZZAQUE, Ph.D.

SPEAKERS PRESENT (Continued):

GERALD E. MARTI, M.D., Ph.D.

C O N T E N T S

C O N T E N T S (Continued)

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P R O C E E D I N G S

(8:00 a.m.)

DR. PURI: If you'll please take your seats, this will begin our second day of the workshop.

An announcement. The company who's making a videotape of the entire program, and if you're interested in getting a copy of the videotape or on CD-ROM, please contact the gentleman in the back. There is a form you just fill out, and he'll be happy to send you a copy. So you could order your entire program in the videotape.

On behalf of the organizing committee, I'd like to thank all the participants of this workshop who have made this first day a very productive conference as I heard from many, many audience, and I hope that today also we have a very full schedule, and it's going to be equally productive as it was yesterday.

I'd like to encourage our audience to please freely participate in the panel discussions in all three sessions which we are going to have today.

With that note, I would like to introduce the moderators for the first session,

Session No. III, this morning, and the moderators are Dr. Philip Noguchi, who's the Director of the Regional Cellular and Gene Therapy Center for Biologics and Evaluation Research, and the co-moderator is Dr. Bernard Fox, who's Associate Professor of Immunology and the Chief, Laboratory of Molecular Tumor Immunology, at Earle A. Chiles Research Institute.

Dr. Noguchi.

DR. NOGUCHI: Thanks, Raj.

As Raj was saying, we do have a full schedule, but I want to just take one or two minutes to, first of all, thank Raj directly for putting together such a wonderful program.

Now, today this first session is really where the rubber hits the road, I think, because what we have said before, you've learned about what FDA does in general. You've learned something about the newer dendritic cell characterization, but when you start putting it into patients, we come back to the same basic things.

First of all, what is it that you're putting in? How can we best understand what you have there? Is there anything that can tell us what when you put that product into the patient, that it

actually is going to have some activity? We're not going to be talking about whether it works or not, but you don't want to just be putting in a bunch of dead cells, as an example, because we really don't think that's an appropriate thing to do.

And then, finally, how can we all do everything that we've done up to now, but do it even better? Because, after all, for our patients, and that includes not just us, but certainly all of the investigators, what you're looking for is really an effect on the patient that will benefit him or her.

Now, that's a big challenge, but I think our opening talk by Dr. Plautz today on a very novel vaccine, which I think you're going to enjoy quite a bit because it is very well characterized, and it does have some nuances to it that are going to be very important in our further discussion.

So if I could have Dr. Plautz, please.

DR. PLAUTZ: Thanks.

What I'm going to talk to you today -- if I could have the first slide -- I'm going to talk to you today about our use of autologous short-term cultured tumor cells as antigens for tumor vaccines, and after all of the wonderful talks we heard yesterday about dendritic cells, the different

preparations of dendritic cells, different ways to load them with antigens, I feel a little bit like I'm trying to sell you a Chevy after you've already test driven a Porsche.

But I think it's important to keep in mind that although we understand quite a bit about how the immune system can eradicate tumors in mice, there's quite a bit we don't understand about how the immune system responds to tumors in human patients, many of whom have had a co-evolution of their tumor with their immune system for a period of months, if not years. So I think that's an important thing to keep in mind.

And a field, I think it's important for us to hedge our bets and look at a number of options, treatment options and collect data on what is a very complex biologic process so that we can evaluate and learn more about the system.

So what I'll try and convince you of today is that autologous short-term cultured tumor cells are useful and that they can provide us some interesting data.

Now, I'll just start with our rationale for using these cells, and the first point is that autologous tumor cells contain unique antigens and

potentially are a source of MHC Class I and Class II epitopes.

I'd like to start with this first point. It's become very clear over the past several years that cancer is a disease of genetic instability. Some of these genetic changes contribute to the transformed phenotype; some contribute to the metastatic potential or potential to recruit blood vessels; but there's also probably a whole host of genetic changes that occur that have a very subtle phenotype and many that that's probably just the tip of the iceberg. There are probably many other genetic changes that occur just as a byproduct of genetic instability, and that these can potentially give rise to unique proteins that can be recognized by the immune system.

Now, that's theoretical. There's also some very hard experimental evidence that carcinogen induced animal tumors contain unique antigens as the immunodominant epitopes, and actually this was first described one year before I was even born by Prenin Mahin (phonetic), and more recently Primad Shivastaves has shown that the antigens carried by heat shock proteins also tend to be unique for different tumors.

And in our own system, using adoptive transfer T cells, we find that the response that is generated in tumor draining lymph nodes is exquisitely specific for the tumor that was used to synthesize, and not even cross-reactive against different tumors of the same histologic type generated in the same litter of mice by the same carcinogen.

So I think this is a very important point that we should not force, that the experimental evidence suggests that the immunodominant antigens are unique to tumors.

The other thing is that autologous tumor cells can serve as a source for Class I and Class II epitopes, and we're very interested in looking at treatment of brain tumors, and what we found in our experimental models is that CD-4 cells are crucial to this process.

And actually under the right culture conditions, CD-4 cells alone, in the absence of CD-8 cells, can eradicate tumors, and the tumor that we use is Class II negative. So I think it's important that we also keep in mind a source of Class II epitopes.

The second point is that unlike the field with melanoma, where a number of very important advances have been made in identifying and characterizing tumor antigens, for many tumors the tissue restricted or shared tumor antigens have just not been identified and characterized at a molecular level. So we just don't have these reagents available for some types of tumors, and especially for the gliomas which we're interested in studying.

So that provides a rationale for using autologous tumor cells. Now, in terms of the point of short-term culture, what we found is that -- and I'll show you some slides to demonstrate this in a minute -- is that the short-term culture can remove accessory cells that contaminate the original tumor digest, and also there's quite a bit of necrotic debris in the original tumor digest that can be eliminated by just a simple process of short term culture.

What I'd like to do is just run you through our protocol so that you have an idea of how we use the tumor cells, the vaccine.

First of all, we obtain tumor samples fresh from the pathologist at the time of surgery. These are enzymatically digested to prepare a single

cell suspension, and we take an aliquot of these cells; we freeze them; and then the remainder are grown for a period of two to four weeks, and I'll describe this step in a little bit more detail in just a minute.

The second step is that the patients are vaccinated for renal cell carcinoma one month after surgery or for the malignant brain tumors after the patients have completed their radiation therapy. The culture tumor cells -- we use the dose of 20 million -- are irradiated and then mixed with GM-CSF as an adjuvant. These are injected intradermally on the upper thigh, and then GM-CSF is injected into the vaccine site for an additional three days.

I'd like to make one comment about the vaccination. Prior to using GM-CSF, we used autologous tumor cells mixed with BCG, and this caused severe ulceration in some of these patients, especially the brain tumor patients, many of whom have some degree of immunosuppression.

And in contrast to that, this is very well tolerated. The mixture of GM-CSF with the autologous tumor cells causes about a two to three centimeter area of erythema and a smaller area of

induration right at the injection site, but it's very well tolerated.

Now, this is due to the combination of GM-CSF and the tumor cells because when we inject tumor cells alone at a distant site for DTH response, here's no erythema, and additionally, when you inject GM-CSF, by itself it doesn't cause erythema.

So it's really this mixture that causes this local reaction.

Now, this also causes a reaction in draining lymph nodes, and we see hypertrophy of draining lymph nodes that occurs over the subsequent week. So nine days after the vaccination, we remove the vaccine sites.

And just one little caveat here. We're using vaccination as one step in a chain of events, and for our purposes vaccination is given as a single injection, and it is used solely for the purpose to sensitize T cells and draining lymph nodes.

So the requirements and the optimal conditions for this type of vaccination may or may not differ from successful strategies for

vaccination for active immunotherapy. So I'd just like you to keep that in mind.

Once we obtained the lymph node T cells, they're dissociated into a single cell suspension and then activated with staph. aureus enterotoxin A, which is a very powerful mitogen for human T cells, for two days and then cultured in serum free media containing IL-2 for an additional five to seven days.

And with the proper culture conditions, we can in most cases get greater than 30-fold expansion of the T cell numbers, and in some cases close to 100-fold expansion over this short period of time.

The patients are conditioned with cyclophosphamide one day prior to receiving their T cell infusion, and this is done as an out-patient procedure. The patients do not receive concomitant IL-2. This is based on our preclinical data in mouse intracranial tumors where we found actually IL-2 was detrimental to the trafficking and efficacy of the T cells. So we just used the T cell infusion alone.

So you can see now how vaccination fits in as a single step in this chain of events.

Now, to concentrate a little bit more, since this is about tumor vaccines, about the exact method that we used to prepare the tumor samples, we obtained fresh samples. They're transported under sterile conditions to a dedicated tissue culture facility, and then necrotic debris and blood clots are removed, and the tumor tissue is minced.

And this is a very important point, especially for the malignant gliomas we worked with. One of the pathologic hallmarks of glioblast homomultiformi (phonetic) is that there's necrosis and vascular proliferation.

So these tumor samples tend to be very bloody, and there's quite a bit of necrosis and debris, and you'll see that in some of the slides coming up in a minute.

And then a single cell suspension is prepared by digestion with a mixture of these enzymes, collagenase, hyaluronidase, and DNAase, and then the cells are filtered and washed twice.

Now, this washing step gets rid of some of the soluble debris that's present in the culture, but quite a bit of necrotic debris does pellet with the cells.

Some of these cells are frozen away immediately after the tumor digests, but the majority are cultured. They're added to fibronectin coated flasks, and then cultured in this mixture of DMEM, ex vivo, 15, which is a serum free media, and then five percent AB serum.

The glioma cultures are also supplemented with this G-5 which contains some selenium transferrin and hydrocortisone and a little additional hydrocortisone, and then we're able to fairly reliably in about 80 percent plus of the cases establish short-term cultures of tumor cells.

And just to address the issue of whether there's long-term selection of different phenotypes, what we do is just use short-term cultured cells. So presumably there's not been a lot of selection that occurs during this short period of time.

Prior to vaccination, again, the cells are given a single dose of radiation immediately prior to their use. So that's how we prepare the cells.

And what I'd like to do is just show you some examples of short-term cultured cells, and many of you in the audience have probably worked with renal cell carcinoma. So this is a patient with

renal cell carcinoma, just to give you kind of a perspective that you're familiar with, and this is the original single cell digest.

And what you can see is there are adherent cells here that have flattened out in this fibronectin coated flask, but there's also some debris in here, and there's some red cell contamination.

Now, two days later we just rinse off the loosely adherent and nonadherent cells, and what you're left with is a lawn of cells, and in here many of the cells are flatted out, but there are still quite a few that are very loosely attached to the fibronectin coated plate.

These are probably dead cells that are just stuck onto the plate. After one passage though, what you see is that many of these cells now have flattened out, and so it's a much cleaner preparation.

And this is just another example of a renal cell carcinoma sample where you can see in the original digest there's in this sample quite a bit of red cell contamination and not so many dead cells, but then quickly the cells establish a

monolayer flattened out, and it gets rid of a lot of the necrotic debris.

Now, this really became much more of an issue when we started working with glioma samples because what you can see here is in the original single cell digest, you can't even see the cells that are attached to the plate. There's quite a bit of red cell contamination. There's also a thick film of necrotic debris that just rests on top of the cells.

Now, a lot of this can be rinsed away, but a lot of the residual cells are still probably dead in this original mixture. After passing the cells, you end up with a much healthier looking culture.

Here's another example showing pretty much the same thing, where there's quite a bit of debris in the original digest. It cleans up, but in this case many cells are probably deal, and when we look at Trypan blue exclusion in the original tumor digest, in many cases greater than 50 percent, some cases greater than 70 percent of the cells, especially for these glioma tumor digest, are Trypan blue positive.

So we don't really care to immunize patients with a mixture of mostly dead material.

The other consideration for brain tumors is one way to induce EAE in animal models is to just smash up a spinal cord and inject all of the myelin. So by cleaning out all of this debris, presumably we're getting rid of a lot of things that could potentially be autoantigens and detrimental even.

So this is what the culture, again, looks like after one passage, and just to give you another example, a similar type of phenomenon. These cells tend to pile up in many cases and form foci, and then just another example, and in this case a lot of the initial cells are dead, are fairly scattered live cells, but they quickly form colonies and quickly proliferate.

So one thing I think that's maybe evident from some of the cultures you've seen, this just looks at four different cultured glioma lines, and then on the next slide, four additional glioma tumor lines, and I think you have an appreciation here. There is quite a bit of variation in the morphology of these cultures.

And we spent a lot of time looking at these cells under the microscope, and when we were

establishing the conditions for growing these cells, we were really impressed with the variation in the morphology, and kind of at a subconscious level for us, it kind of reminds us that there's probably quite a bit of heterogeneity in antigens in these different tumor cultures as well as the variations in the morphology.

So since this is a workshop, what I thought I would do is just touch on a couple of the points that I think are important.

First of all, what would be necessary qualities of an autologous tumor cell vaccine? Well, of course, we want sterility in terms of gram fungal in cultures, endotoxin negative.

Another thing that I think is quite important is that we have intact cell membranes. In animal models it's quite clear that if you break the cell membrane and inject that and try and use that to generate vaccine draining lymph nodes, that it doesn't work very well.

So it's important really to have Trypan blue excluding cells, viable, healthy cells in the vaccination mixture, and when we harvest these cells from tissue culture flasks, routinely they're 85, 90 percent-plus Trypan blue excluding cells. So they

seem to be in a bit healthier state compared to the original tumor digest.

The third point that's important is that they should have an inability to form tumor, and we use irradiation to prevent these cells from growing.

We followed many of these patients out now past a year after their vaccination, and we've seen no evidence that there's any tumor growth at the vaccination site. So whether the irradiation kills every last single cell or whether just the injection site intradermally is a poor substrate for these tumors to grow.

We have not seen any problems with tumors due to the vaccination procedure, and then, of course, the certificate of analysis for all of the reagents used in the tissue culture.

Now, these are necessary qualities. When I was thinking about what would be ideal, in an ideal world what would be desired properties of an autologous tumor vaccine, it would be very nice if we could have some way to document the tumor phenotype of cells that we grow out in short-term culture.

Now, this is, I say, in an ideal world because practically speaking, as you've seen, I

think, from the slides I've showed you, we can't really use morphology as the criteria because the morphology is highly variable. Some types of tumors contain cell surface markers which are convenient to use.

We've tried looking at GFAP in our brain tumor models. That's not routinely expressed. We've looked at tolemerase (phonetic). We see it in many cases, but, again, it's not uniformly expressed.

So this is a bit of a conundrum for us because in many tumors there doesn't seem to be a uniformly expressed marker that's easily tested at the time that you give the vaccine.

Another approach would be genomic, genotypic characterization and identity with the original tumor specimen. Many tumors contain genetic abnormalities and perhaps through a use of comparative genomic hybridization or spectral karyotyping, some of these newer genetic approaches, it would be possible to document that the tumor cells that are used for the vaccine are identical to the tumor cells that are removed from the patient.

The technology here, I don't know if it's developed to a point where it can be routinely

used in an easy manner by a number of laboratories. So I think this is something that may have to wait until the technology develops and is a little bit more accessible.

And this last point, again, is a bit of a conundrum for us because, of course, you'd like to have some type of functional test that what you're injecting into the patient is immunogenic. The problem is that these are unique vaccines for each patient, and when we test these patients before they're treated, we really don't see any T cell immune response. So how can you test something that's nonexistent before you administer the vaccine?

We have plenty of post hoc evidence that the cells we get from the lymph node respond to autologous tumor by production of gamma interferon and other types of measures, but again, prior to the procedure vaccination, it's very difficult to do a functional test on each individual patient.

So these are some things that, you know, in an ideal world it would be nice to have, but we're sort of limited in our ability to satisfy all of those potential requirements.

So in conclusion, what I'd like to say is that short-term cultures of autologous tumor cells can be established for most patients, and we've been more than 80 percent successful in patients with malignant gliomas and renal cell carcinoma.

This is a big advantage. The cultured tumor cells are free of debris, and they're also free of accessory cells. I didn't show in the slide, but we've tested some of them, tumor digest, and for renal cell carcinoma, many times there's greater than ten percent CD-14 positive cells in the original tumor digest. There's a number of CD-31 positive endothelial cells in the original tumor digest, and when we test the cultured cells, these disappear.

So it helps to get rid of these accessory cells, and this can be done during short-term culture.

And then the procedure of vaccination with irradiated culture tumor cells is associated with minimal toxicity. We've treated close to 60 patients now, and really there's minimal toxicity at the vaccine site.

And what we observe is that there hypertrophy of vaccine draining lymph nodes, and we've tested -- we haven't tested every patient, but when we've tested, we see that there is reactivity against autologous tumor from T cells in these draining lymph nodes. So that's one immunologic measure that there is some response.

And then we've also, in our Phase I trials, seen some clinical responses in patients. So, again, this is a harder piece of evidence that patients treated with ex vivo activated lymph node T cells, that these culture tumor cells are sufficient immunogen.

So I'd stop there and thank you.

(Applause.)

DR. FOX: Thank you, Greg.

I'd like to now call on Dr. Ernie Yankee, who will give our next presentation. Ernie Yankee is the Executive VP of AVAX, and before that he was at Upjohn, and he has responsibility for all of the R&D and regulatory affairs at AVAX.

DR. YANKEE: Dr. Noguchi, Dr. Fox, on behalf of AVAX Technologies, I want to thank Dr. Puri and the other members of the organizing committee for the opportunity to present.

This meeting is very timely and especially important not only because of the large numbers of novel technology product INDs currently at the FDA, but also the large number of companies developing novel technology products.

Especially this meeting is important because of the many thousands of patients who need new therapies.

Successful development requires the productive cooperation between science, industry, and the regulatory authorities. However, as evidenced by this workshop, novel technologies challenge the existing regulatory framework.

I want to first discuss where the existing regulatory framework can accommodate characterization of our novel technology product, but the bulk of my talk will be spent discussing the challenges that novel technology products like ours face in dealing with the historic framework, and of course, I'm going to provide a proposal on how we think we can deal with one of those challenges.

Next slide, please.

We view that in the area of emerging technologies, we view FDA's role to be not only the traditional insuring safety and efficacy and

preventing fraud, but equally importantly, assuring regulatory flexibility.

This provides a bit of a challenge to the agency because they need to balance regulatory flexibility with also providing clear and consistent guidance.

This meeting also provides the opportunity to aid in development of policy to avoid podium policy and provide a level playing field for all of us who are trying to develop these novel products.

Next, please. Back, please.

Probably most of you know about these guidelines, but I want to just review very briefly the agency's criteria for premarket review for cellular based products.

There are four key components of this: more than minimum manipulation; combination with a non-tissue component; used for a nonhomologous function or used mostly for metabolic function.

The first two are probably most relevant to my talk today, and we view the second one as a combination with nontissue component, although not explicit in the guidelines, as encompassing the

combined therapeutic administration with adjuvants in other agents, in our case today BCG.

Next, please.

These are some examples of the distinction made between minimal manipulation and more than minimal manipulation. On the latter, more than minimal manipulation for our purposes is most important with regard to change in biological characteristics of the product, and I've given some examples of where this would be in the latter case.

Next, please.

Our product is a Hapten-modified tumor vaccine. It's autologous, and it's intended for patient centered therapy.

There is cell manipulation with a potential change in biological characteristics through the Haptenization procedure and through irradiation.

It is combined with a noncellular component, namely, Hapten modification and administration with an adjuvant, BCG.

Next.

There are a number of challenges inherent in the nature of our product. The shelf life provides a limited window for product release

testing. Because it's an autologous system, for the purposes of lot release it's an n of one.

The immune response which we want from our product requires in vivo induction. The correlation with any in vitro assays is obviously a very serious challenge.

Finally, sterility can be a challenge if the vaccine is manufactured from a likely non-sterile tumor source.

Next, please.

Our experience leads us to believe that central manufacturing is far superior to multiple site manufacturing because of a number of advantages. Centralized manufacturing provides decreased variability both in manufacturing and in validation, and it minimizes the need for demonstrating bioequivalence for products prepared at different sites.

Next.

In meeting the challenges of the regulatory framework with this novel technology product, we have gone a considerable ways. We follow good tissue practices, for example, including patient screening for communicable diseases.

We have in process reagent solvent removal with validation or established finished product specification, and we've established the nonproliferation of the tumor cells.

Next, please.

We've also demonstrated the efficiency that Haptenization with appropriate process controls or finished product characterization. We have measures of cell viability and morphology both in process and finished product specification. We have an endotoxin assay, and we test each lot, and we don't do a general safety test.

Next, please.

Sterility is tested on every lot and results, of course, are reported after administration because of the shelf life. This is an example of where the agency has been very flexible in trying to help work with an area like this.

We have identity assay which we are using, which is a combination of measurement of expression of melanoma cell antigens, as well as anti-Hapten antibody measurement.

Next, please.

As you've seen, products such as ours can meet the existing regulatory framework for characterization. Nevertheless, challenges remain, the largest of which is the potency assay.

Product specific potency assays are required for lot release according to the regulations, and the regulations define what potency means, which I've indicated here.

I want to just point your attention to the part that we have italicized from this: appropriate laboratory tests, adequately controlled clinical data.

Next, please.

Dr. Noguchi in a 1996 publication reviewed this area and indicated that potency as a measure of clinical usefulness was added over 50 years ago to the Public Health Service Act. The implications for patient centered therapy are that the extent of characterization should be consistent with focus on potential clinical utility.

Next, please.

I've listed here the attributes that are common with the potency assays, that is, for traditional small molecules and biologics used to characterize the product to monitor consistency, to

assure stability. Results are available prior to release. Close relationship to the putative physiologic-pharmacologic activity of the product, ability to elicit a dose response, and the ability to validate the assay.

Potency may also be measured then in animal model and/or functional assay performed in vitro or in vivo.

Next, please.

There are two key conceptual challenges for a product like ours in dealing with a potency assay. First, an animal model is not an intuitive option, and more to the point, it's simply not available.

Secondly, and more to the point for what we're going to be presenting later this morning, can the ability of an autologous product to induce an antitumor immune response in vivo be measured in vitro?

We've spent a great deal of time thinking about these problems, and we've invested a fair amount of research trying to address them, and I want to summarize in the next two slides sort of where we are with what we have found in our thinking.

Next, please.

Potency assays can be sort of arbitrarily differentiated between preadministration and post administration assays. Prior to administration assays, for example, could be an allogeneic T cell stimulation measuring as an endpoint either cytokine release or cell proliferation.

Clearly this has a big advantage because it can be conducted prior to lot release of the product. However, there are very serious limitations to this.

The mechanistic relationship to the activity of the product; it's very difficult to validate for an autologous product. Hapten modification has been shown to decrease the response of an allogeneic assay with these endpoints, and the question of whether this is related to clinical efficacy is a very big question.

Finally, we get to the point of what is the value added in even trying to conduct such assays.

Next, please.

Post administration assays, two examples are an autologous T cell stimulation after a round

of immunization, taking cells from the patient. This was published in 1995 with this vaccine.

The other one is a measurement of a DTH response, again, after a round of immunization with the vaccine, measuring DTH response to each of modified and unmodified tumors cells.

The advantages to both of these are there's a close mechanistic relationship to the activity of the product, and there may be potential for predicting or correlating at least with clinical utility.

There are serious limitations, again, here. Inherently these are post release, post administration assays. In particular, DTH assay is highly susceptible to false positives and, by the way, false negatives.

And finally, the assessment of DTH reaction is highly operator and technique dependent, and what I don't have up here is all of these assays put a great burden on the patient.

Next, please.

We want to propose what we think might be a reasonable alternative to these, that is, that the potency assay should be identity assay plus, that is, the identity assay which I've described to

you before for this product, plus, the plus meaning in conjunction with cell viability.

This would insure relevant potency measure prior to administration, and it would correlate. Ultimately one would correlate this with critical measure obtained in the Phase III studies.

There are precedents for this. Traditional vaccines, for example, Varivax, quantitate live virus via the plaque assay. Other products such as Carticel, where cell count and viability are used as the potency assay for release purposes.

Finally, I want to summarize where we are.

Next, please.

The vaccine that we are developing is a novel autologous therapy. As such, the regulatory framework to address this needs to be not only scientifically rigorous, but both flexible and creative.

Identity plus, as we have proposed, meets the potency assay requirements of the regulations. There are appropriate laboratory tests, and there will be adequate clinical data, and it's also consistent with precedence.

Thank you.

(Applause.)

DR. FOX: Thanks, Ernie. That's great.

Okay. Our next speaker is Dr. Jim Mule. Jim is the Maude Tulane Professor of Surgery at the University of Michigan, and he's been very actively involved in adoptive immunotherapy strategies over the last 20 years.

Jim.

DR. MULE: Thanks, Bernie.

I'd like to begin by thanking the organizers for the meeting for the invitation to share with you today some of our most recent information on using tumor lysates as a way of pulsing dendritic cells to serve as an immunogen both preclinically as well as in some recently initiated Phase I clinical trials.

If I could have the first slide, please.

Okay. The hypothesis for this work is the fact that a potent DC, as you've heard yesterday and early today, as antigen presenting cells may uncover in cancer patients very low level activity or T cell reactivity to poor or nonimmunogenic tumors that are virtually undetectable by other methodologies.

Now, why use tumor lysates? Again, yesterday you heard the possibility of using fusion of whole tumor cells by Don Kufe, as well as the possibility of using apoptotic tumor cells as a way of presenting antigens via dendritic cells.

And whether or not lysates are as efficient as peptides or these other methods is something that I think we really need to pay considerable attention to and design appropriate preclinical studies comparing the different ways of manipulating dendritic cells with these different sources of tumor antigen.

But for the purpose of the talk today, I'm going to share with you the reasons why I believe lysates have certain advantages, and of course, if someone were to give a talk with defined peptides, I'm sure that the advantages would be clear in those cases, as well.

But nevertheless, from our perspective using tumor lysates allows a greater potential for augmenting a broader T cell response, given the fact that presumably tumors express multiple tumor associated antigens on the cell surface.

By doing this, the possibility is to lessen the potential for tumor escape from immune

recognition. I think some of the recent peptide studies clear that immunity can escape, can be overcome by antigen modulation on tumor cells.

A greater potential to trigger T cell reactivity to tumor rejection antigens, obviously to date there's been a number of peptides molecularly cloned, particularly in melanoma, and more information it's now becoming clear from the clinical trials that give us an indication of whether or not any of those peptides are defined as classic tumor rejection antigens.

And then lastly, the fact that lysates may allow you -- and you'll see from some of our work in the mouse -- allow one to generate a greater potential for presentation of both helper and CTL defined epitopes.

Now, from a practical standpoint, the use of lysates allow the following advantages. One is that one can use crude lysates, and it becomes an issue of how one defines these tumor lysates, which we can talk a bit more during the panel discussion perhaps.

But nevertheless, it allows us to circumvent the need for viable fresh tumor cells

since we use crude lysates of freeze-thawed three cycles and use that to pulse dendritic cells.

Obviously it avoids the necessity of molecular characterization of tumor antigens. That becomes a critical issue when one recognizes the limitations to date in trying to identify tumor peptides associated on histologically distinct human tumors that are distinct from melanoma, as an example.

And then lastly, it's becoming more clear that CD-4 responses are playing a significant role in the antitumor response generated, and that one very much needs to take into account the necessity for help in any of these vaccine strategies.

We showed years ago in this mouse model that one could readily take a sarcoma 207 and post the lysate onto dendritic cells and in vitro bring out a specific proliferative CD-4 response and in a crisscross experiment in parallel using a variety of different tumors, such as a colon cancer or the Lewis lung cancer, essentially one could show exquisite specificity of the proliferative response of CD-4 cells when one uses crude lysates post onto DC.

We then moved on to using bone marrow derived dendritic cells for the remaining murine studies that I'll discuss. This shows the classic slides of dendritic cells generated in GM-CSF plus IL-4 using whole bone marrow from mice.

And clearly these cells, as you've heard yesterday from Ralph Steinman and Jacques Banchereau, are very potent in their ability to stimulate primary aloe responses. This is one example in which Metrizamide separated dendritic cells from the marrow could trigger very powerful MLR compared to the pellet from the Metrizamide gradient, and in every indication that we have in these assays, the proliferative potential induced by dendritic cells surpasses manyfold what one can achieve with the optimum amount of CON A (phonetic) stimulating those T cells in culture.

This just shows a battery of antibodies that one can use to show we have dendritic cells. They're high class 286, 80, 40 CD-11C, but do not express B-220, and this is the pellet from that gradient.

We showed in a paper that was published some months ago that in vitro one could educate CTL by taking naive spleen T cells, incubating or

stimulating those T cells with lysate pulsed dendritic cells in the presence of low dose IL-2 plus IL-7, and one can show specific induction of CTL by using lysates against the 207 tumor, but not an irrelevant sarcoma 102.

We then showed that this response was Class I restricted by using the appropriate haplotype specific Class I antibodies. We could essentially eliminate that CTL activity.

And also, the CTL could generate in a specific way when triggered in vitro by the appropriate lysate post DC GM-CSF production compared to controls.

So we were, in fact, able to generate in vitro specifically reactive T cells by using a lysate. Now, obviously one needs to show in in vivo models that what we have will impact to some degree on tumor, and what we then did was to move on to in vivo experiments.

Here is one example within a syngeneic MT-901 breast tumor in which we immunized mice with lysate pulsed DC and then rechallenged those animals with large amounts of friable tumor cells. All animals were protected as one would expect compared to the control groups.

We then went on to show, using an early established model of pulmonary metastases. In this case, these data are with an MCA-207 sarcoma, but we've also done this with the mammary tumor as well, that the use of tumor pulse lysate DCs administered subcutaneously to mice that had three-day micro metastatic disease in the lung could substantially reduce the number of metastases.

This shows the number in the lungs versus the treatment groups, and more importantly, we showed that if one were to deplete the animals selective at CD-4 cells or CD-8 cells, that that impacted significantly on the ability of this immunization procedure to cause regression of these micro metastatic nodules.

So clearly the effect was mediated by T cells. It was mediated predominantly by CD-8 cells and CD-4 played a participatory role as well.

We've moved on to a Phase I clinical trial based on those preclinical animal studies, and we're in the midst of the Phase I trial. This cartoon shows the approach.

We take fresh tumor, prepare a lysate ahead of time. It's characterized by sterility and so forth, and then the patients are leukophoresed

for four hours and dendritic cells are generated in the standard way from monocytes using GM-CSF plus IL-4.

We pulsed the lysates overnight, and then the patient receives interdermal injections of this pulsed dendritic cell over time, and we then monitor the peripheral blood for response.

This gives a little bit more specifics about the trial. It's a dose escalation Phase I in which half the number of dendritic cells are pulsed with KLH. Half are pulsed with the tumor lysate. They're mixed and injected. The lowest dose is one million cells.

We're now in the midst of the ten to the seventh dose of this escalation, and were approved in a separate cohort of six patients once we reached the highest dose level to evaluate the capacity of tumor pulsed DC to sensitize draining of lymph node T cells.

As I said, in the patients we're now very early in the analysis, but I'll show some preliminary data. We have used LDA looking at proliferative T cells, and if one does pre versus post PBMC looking for a response to tetanus in these patients, as you would expect, there's no difference

pre versus post in the frequency of T cells in the periphery of these patients.

However, after immunization with KLH, we're now able to show in all patients that the ten to the seventh dose so far with this LDA assay, a skewing or biasing or the frequency is shown here, which in most cases represent a frequency similar to what the patient is showing with tetanus toxoid.

And we skin test the patient one month after the last immunization. Before we skin test, we take a two-hour leukophoresis for the immune assays.

This shows the patient that was skin tested post immunization at one microgram, ten micrograms, and 100 micrograms of KLH, comparing that DTH response with tetanus toxoid in this patient.

In a patient, the first patient at the ten to the seventh dose, we've seen a partial response of melanoma in this periodic (phonetic) lymph node. We've now gone on to retreat this patient with a second cycle of immunizations at the ten to the seventh dose.

The patient has now received the second immunization of the second cycle.

Now, we're also interested in where we go once the Phase I is completed. So what I'm going to share with you now are some new data that will be appearing in the PNAS in the next month or so in which we, based on the fact of the data that were shown to you that T cells play an important role in the antitumor effect mediated by lysate pulsed DC immunizations, it was clear to us that IL-2 may be a cytokine of value in augmenting the T cell response.

So what we did was to use IL-2 doses in the mouse that were 25 to 50-fold below the MTD, and if one extrapolates to humans considering the MTD in patients, this dose, perhaps with a number of caveats, would represent doses that one would call T cell reconstituting doses based on the studies of Calogary (phonetic), for instance, in HIV and Alex Pfeffer (phonetic) with patients undergoing pulmonary transplant.

But what you see here are treatment of pulmonary metastases that were established at three days versus those in which immunizations are delivered at day seven. One grossly visible tumor is seen on the surface of the lungs.

In this case, tumor lysate pulsed DC alone have a small effect. However, as we published

earlier, on three-day micro metastatic disease, the effect is quite considerable.

However, IL-2 at low dose administered in a three-day cycle after each immunization gives you a significant antitumor effect over that achieved by tumor pulsed DC alone, which is more significantly seen when one goes to a more established tumor model.

It was interesting to us that animals that were actually cured of disease at seven days -- we followed these animals out for at least 100 days -- if we took spleen cells from those animals at about two weeks after tumor -- we believe tumor was cleared from the lungs of those animals, we were able to show in vitro that those T cells could selectively secrete gamma interferon in this particular experiment on the level of 250 units, with low level of activity against the controls.

Given that information, we went to a B-16 melanoma model, and this tumor is a subline of B-16 melanoma denoted D-5, which has very low level Class I expression, no evidence of Class II expression, and other antibodies are used here.

But as was published by Manson Seay (phonetic) at Harvard and a number of other

investigators, CD-44 is a marker for metastatic potential of these cells. These are highly aggressive, highly metastatic. This is a highly metastatic subline of the B-16 tumor, and again, it's low in Class I.

When we incubate in culture, not surprisingly this line with 200 to 300 units of gamma interferon, we can up regulate Class I, and again, those are the levels that we've detected in T cells in vitro that are incubated with lysate pulsed DCs to trigger those cells to produce gamma interferon in animals that are treated with lysates plus IL-2.

We can treat B-16 D-5 in a three-day model. We're now moving on to more established models by combining tumor pulsed lysates, tumor lysates pulsed to DC, combining that with IL-2, and here is an experiment in which IL-2 alone has very little effect; lysate plus IL-2, no effect. The controls, the other controls are shown.

Lysate plus IL-2 at three immunizations will impact to some extent. It's not great, but you can see here a significant antitumor effect when one combines the lysate pulsed DC with low dose IL-2 administration.

So we're now in the midst of submitting to the IRB a clinical protocol which will allow us to combine IL-2 with DC based immunizations.

I'd like to finish by telling you another strategy that we're involved with and have IRB approval to go ahead with, and that is to combine DC immunizations in the Pulmonary Transplant Center.

We have a dedicated facility in our 16-bed clinical research center at Michigan, a dedicated facility that allows us to perform leukophoreses. We also have a dedicated set-up for CD-34 stem cell isolations. This is the Baxter 300I separation device.

We've completed a number of studies in the transplant unit of giving -- successfully reconstituting patients with selected CD-34 cells off the column, and given that information, you've heard from Jacques Banchereau, as well as Ralph Steinman, yesterday the potential of generating dendritic cells from CD-34 cells.

All the data I've provided so far were with the monocyte derived dendritic cells, and we're now pursuing comparisons between negative fractions off the CD-34 column, comparing the activity of

dendritic cells generated by this negative fraction compared to the highly purified fraction off the clinical column, and these will be done by in vitro assays.

Another important aspect of this work is that from a single leukophoresis collect or pool collects, depending on the number of cells that are needed for the transplant, one can obtain both a negative fraction to generate large numbers of dendritic cells for immunization post transplant, as well as having grafting dose of purified or highly enriched CD-34 cells for the transplant.

And this shows a trial that we've started not with tumor lysate pulsed dendritic cells, but using KLH as a marked antigen pulsed onto dendritic cells in which we're immunizing non-Hodgkin's lymphoma patients, intermediate grade, with dendritic cells pulsed with KLH, starting at lymphocyte counts of 500 post transplant, and comparing that to patients being immunized with KLH alone, and that will be an immunologic pilot study to determine whether or not we can bias or educate the developing response early on post transplant.

So I'll stop here and thank my many collaborators within the Department of Surgery, the

bone marrow transplant group within the Department of Internal Medicine, Paul Watkins who's the Director of our GC-RC and Sandy Hoffman in the Blood Bank.

Thank you for your attention.

(Applause.)

DR. NOGUCHI: Now, yesterday Dr. Zoon said that when she started at the FDA tumor vaccines had been around for a while. Well, I've been at the FDA maybe ten years longer, and that statement was true for me as well.

And perhaps even longer than that, Dr. Morton is really one of the pioneers in this whole field, and we're very pleased to have him here today because I think Don has been able to not only initiate and start this very exciting field, but has been able to move and to evolve with new technologies.

So Dr. Morton.

DR. MORTON: Thank you, Phil.

You know the definition of "pioneer" is somebody who's lost in the wilderness.

(Laughter.)

DR. MORTON: But it is really very exciting for me to be here and to see 500 people at

a meeting on tumor vaccines. Even a decade ago when you got up to talk at the medical oncology meetings, why, all of a sudden everybody would go to the exhibits, and --

(Laughter.)

DR. MORTON: -- and it's just amazing.

I really want to tell you that I know this is an important field today because if you go through the attendance list and see all of the attorneys in the audience, you know that --

(Laughter.)

DR. MORTON: -- tumor vaccines have arrived.

Now, this is, I have to say, very nostalgic for me because in this very building 30 years ago, we began our first experiments with autologous vaccines, and it's been a long, long time, but we're very excited that the FDA and the NCI have put this conference together. Dr. Raj Puri, thank you for inviting me.

So I'd like to just review some of the conceptual. These studies that go back to the '60s we asked the question: in asyngeneic animal models, first in mice and then guinea pigs, what's the most

efficient way to immunize against the already implanted and growing tumor?

And we looked at a number of things, but we found irradiated tumor cells mixed with microbacterial adjuvants were the most effective immunogen, and these had to be living tumor cells. Dead cells or cell lysates or purified antigens in this model didn't work.

So we went, after failing with autologous vaccines, we asked ourselves: well, could we use allogeneic vaccines grown in tissue culture?

And from '71 to '84, we tested three different combinations of randomly selected melanoma cells based upon their ability to grow in culture, mixed with BCG, and we saw no clinical responses, and when given in the adjuvant setting, no overall survival effect.

And we were really about ready to give up this approach when one of our postdocs. working in Dr. Rako Erie's lab found that the patients that formed IgM antibodies exhibited prolonged survival, and the problem was only one-third of the immunized patients developed such a response, but those that did had a 90 percent five-year survival.

So for the T cell chauvinists in the room, I want to say but before T cells there were antibodies, and --

(Laughter.)

DR. MORTON: -- and to say that tumor antigens were discovered with T cells is not quite correct. In fact, the work in our laboratory by Dr. Rako Erie suggests that both T cells and antibodies can recognize the same antigen, in fact, the same decapeptide. And so let's not ignore antibodies.

Now, after these failures, we said, well, we've got to go back and reengineer our vaccine, but by this time we had identified in our laboratory six antigens that were immunogenic in man and induced an immune response, an antibody response.

And so we went back and selected from our 150 melanoma cell lines three that had high concentrations of these six antigens, which then were pooled, cyropreserved, irradiated, go through quality assurance and quality control, and then we used as a vaccine.

This vaccine has multiple antigens. All of the ganglioside antigens, the myelinogenesis antigens, and a whole host of protein antigens, and

we've shown that we make antibody responses, in fact, to all of these antigens.

Now, the importance of a polyvalent vaccine is shown by this experiment of nature that Bob Goode used to talk about. This is a patient with no metastasis in which I did a groin dissection, and here you see a clone of melanocytic cells.

Here you see an amelanotic clone in a different lymph node, and here in the same lymph node you see melanotic, amelanotic, and a gray. You can see phenotypically the heterogeneity that exists in all cancer, and therefore, we have to have a induction of a polyvalent response.

So because it's more difficult for tumor cells to modulate or delete multiple antigens simultaneously, even though they are genetically unstable, and the induction of cytotoxic antibody is very important because it's not susceptible to HLA modulation by which to escape the CTL.

Now, in looking at the regulatory aspect of this, the fact is the cancer vaccines have no direct cytotoxic effect on tumor cells. It is not like a drug. So you give the vaccine to a patient. They haven't had the effective therapy until they

induce an immune response to antigens shared by the vaccine and tumor target cells.

And this is some examples of our vaccine. This is antibodies to MAGE-1 that Dr. Dave Hoon's laboratory -- and here you can see two out of the three patients respond.

The purpose of this is to emphasize that there's heterogeneity not only in the tumor, but in the outbred human population that you're immunizing. So one patient will respond to an antigen; another one will not.

And here's another response, an IgM antibody to TA-90, which is a very important antigen is cancer.

Now, of course, it's necessary to induce reactivity with the allogeneic vaccine that cross-reacts with the autologous tumor, and this is lymphocytes co-cultivated with tumor cells and a mixed tumor-lymphocyte reaction at baseline versus 16 weeks later.

As you can see, we get stimulation. It varies from patient to patient, but we get enhanced thymidine incorporation stimulation with the autologous tumor.

Dave Byrd has emphasized he lymphocytic infiltrate into tumors. Metastatic tumors typically don't have T cell infiltrates. Here you can see a patient with a pulmonary nodule that after vaccination stood stable for 12 months, and finally I got tired of watching it and took it out, and this is what it looked like.

You can see you hardly see tumor cells there with the T cell infiltrate.

We also in fortunately few patients induce melanoma associated hypopigmentation, that this occurred about two months after the patient was immunized.

So to understand how vaccines work, the immune response adduced must be studied. The vaccines can only work in individuals who mount an immune response. Knowledge of what constitutes an effective antitumor immune response then will guide selection of QC assays.

And this is the development plan that we developed for our vaccine, Cancer VAX, which we abbreviate in the slide C-VAX.

First, we test it in Phase I-II trials looking for clinical activity. We think that the rule that you have to show some evidence that the

tumor goes away is a good one, and then once -- because we're not really just here to induce immune responses. We're here to induce immune responses that work.

And then we determine which of these immune responses to which antigens correlate with the clinical activity and develop lot release assays based on these antigens, produce lots of vaccine based on these lot release assays, and then test these in Phase II trials for their consistency in inducing immune response to important tumor antigens.

And only after we had done that that we saw it was time to begin Phase III trial.

Well, does this vaccine work? In people with in transit melanoma -- and this was a specifically selected model. As Dr. Keegan said yesterday, the problem is it's asking a lot to expect a vaccine, the host immune response, to take care of a pound of tumor, but people with in transit disease, you can detect small amounts of tumor, and in 54 patients we've immunized, we got 13 complete regressions. Four of those are still in complete regression 22 to 105 months later. There's been no relapse in the CR sites.

Now, people say, well, melanoma spontaneously regressed. Well, I can tell you I have treated over 8,000 melanoma patients in my career, and I've seen two spontaneous regressions. So the incidence is very, very rare.

The other thing we have done is looked at giving this a post surgical adjuvant, the other model that Dr. Keegan mentioned, and we have highly significant prolongation.

Now, in addition to the heterogeneity in the patients and their ability to respond and the heterogeneity in the tumors in terms of their expression of antigens, we have the heterogeneity in the tumor burden in the patient.

And if we look at the level of metastatic disease, whether it's low or high, and the level of antitumor immune response, whether it's low or high, you can see that if you have a high level of metastatic disease and a low immune response, you don't do well. If you have a low level of metastatic disease and high antitumor immune response, you do very well.

So there's this other factor that's going on that has to be taken into consideration.

Now, here's a patient with multiple in transit disease, the failure of radiation therapy and surgery. These are hypothermia burns. Four months later lesions begin to fade, and four years later this patient is still in complete remission, almost nine years now.

We see responses occasionally in visceral metastasis. As you know, it takes a certain size tumor to be detected. This is a two and a half centimeter liver metastasis. This is five months later. This patient is still in complete remission almost five years.

Now, going to Stage IV disease, in our institution there's been absolutely no progress in the treatment of Stage IV melanoma over the last 25 years. As you see, the median survival has stayed the same.

However, in those patients that one can resect a distant metastasis, we do have, in fact, a median survival of 17 months and a 15 percent five-year survival.

But in 150 patients that we resected their distant metastasis and then gave them this vaccine, we have a 39 percent median and 42 percent five-year survival. I was shocked when our

statisticians analyzed the data because, you know, as you're taking care of these patients on a daily basis, you remember your failures more than your successes, but this is extraordinary.

Now, our statistician said, "Well, you are selecting the patients. You know which one's going to do well. So, therefore, you select those."

So they did a matched pair analysis where they took the important prognostic factors for Stage IV melanoma and then matched them with the vaccine patients by gender, site of metastasis, and number of involved organ sites, and again, the data, median survival 36 months, five-year survival 40 percent, and the control.

So it's very clear that there was no selection going on here, as best we can tell, without proving this in a randomized trial.

Now, we asked the question: what are the immune responses in this population that are important for a clinical effectiveness? And we have looked at 77 of these patients and then to correlate the specific immune response in patients receiving this vaccine.

The antibody we're measuring is TA-90, as shown here, glycoprotein. It's present in 72

percent or more of melanomas. It's autoimmunogenic, and we get IgG and IgM antibody responses to it.

Now, in order to say that the immune response to a particular tumor is specific, you need to have a control antigen, and since we immunize with BCG as part of an adjuvant, we have the BCG as the control adjuvant, and we then measure antibodies to PPD as a control and DTH to PPD as well as to the vaccine.

Well, the correlations are really remarkable. If you have both an IgM response and a DTH response, in these people with resected Stage IV melanoma, 76 percent median, 75 percent five-year survival.

If you have one or the other, 32 months, 36 percent, and if you have no response, 19 months eight percent, this is really equivalent to the group of patients that had no vaccine, just surgical treatment alone.

And by multivariate analysis, the PPD response, either antibody or DTH, has no correlation with clinical course, but the specific antibody response both by univariate and multivariate analysis is when all of the prognostic factors taken care of is very significant.

Now, we have also looked at other antigens. GM-2, and again, we see those that make high levels of antibody to GM-2 do better than those that don't.

We make antibodies to GD-3, the same thing. Both of the gangliosides, as well as GD-2, seems to be important.

So going back on our development plan, we have gotten to this point, and then we need to develop lot release assays based upon this information.

So theoretically the quality control tests assure lot consistency, should reflect those characteristics which correlate with the effectiveness of that particular vaccine, and what those tests are are going to be different for different vaccines depending upon their nature.

Now, for our vaccine, we know antibody responses to Ta-90, to the ganglioside antigens. We have shown the skin test responses. We've shown that MLTR correlates with DTH, and we've shown the induction of cytotoxic T cells to allogeneic haplotype matched and autologous tumors correlate with clinical course.

So we then have selected the following for quality control. Viability, and these are the publications that describe the studies which I don't have time to go into.

HLA expression, we have a haplotype match so that our melanoma, viable melanoma cells can direct antigen present to 95 percent of the Caucasian population, and then we have to depend on indirect antigen presentation by host APCs in about five percent of patients.

Antigen expression, TA-90, DC-100, GD-2, GD-3 and GM-2 were all looked at. We have developed in vitro potency assay based upon cytokine release and the identity of the cell lines by DNA type.

This shows the ganglioside profile GD-2 and GD-3 of the three cell lines and of the final mixture of the three, and this is done so that the percentage of each of these antigens the final vial should equal the individual cell lines.

And with GP-100 we have the three cell lines and then the mixtures. Notice that one of the cells does not carry GP-100.

The in vitro potency assay, it shows a dose response to GM-CSF, and if we kill the cells by heat at low temperatures, they're still intact, but

they don't stimulate in this assay, and confirming the viability of our particular vaccine is an important aspect.

The FDA has been ruthless in insisting we had to have a quantitative antigen assay, and Dr. Gupta's laboratory finally developed this assay for TA-90. It shows different lots of vaccine, the values obtained by three different technicians on all of these lots, and the mean, and we really have, I think -- are there with a quantitative antigen assay.

So allogeneic vaccine to be effective must induce response to tumor antigen in a high percentage of patients, and the ability to induce the response must be consistent over time and among different vaccine lots.

And does our vaccine do it? Yes. Phase II trials demonstrate consistent in vivo activity to these criteria and the survival correlates with the immune response function.

This is the skin test response to different lots of vaccine in two-week intervals, and as you can see, every lot of vaccine induces a good skin test response and a good IgM antibody response. When you see variations, low responses, they're

usually small numbers of patients, and it gets at the problem of the heterogeneity in the immune response.

And this is a prospective study, testing this, but we see the same thing as we see in the retrospective studies, that is, those that make both antibody and DTH do better than those that make either, and notice that only six out of some 70 patients did not make either, and with Stage III disease we see the same thing.

So the vaccine then has been through these steps that we thought were important to Phase I-II trials, that complete regression of metastasis seen, prolonged survival as a post surgical adjuvant. In Stage III and IV melanoma, we've compared to matched controls. We retrogressed prospectively, compared the antibody responses to specific antigens and the cellular immune responses in regard to clinical course.

We developed QC and QA lot release assays based upon clinically relevant product characteristics. We've produced a vaccine based upon these assays.

The test of vaccine in Phase II trials for their ability to induce consistent immune

response to clinically relevant antigens and have evaluated in prospective trials the relationship between the specific immune response and clinical results.

And then finally, an issue began this year, Phase III trials of the vaccine as a post surgical adjuvant, and here you can see Stage III melanoma stratification factors randomized to BCG plus C-VAX versus BCG plus a placebo.

Now, this trial began as an equivalence trial in which we had interferon over here, but with the recent data on interferon we thought that it was no longer a good equivalence trial. So we switched it to an efficacy trial.

For Stage IV melanoma, we resected just the metastasis, randomized on the number of lesions, and the same parallel format.

In closing, I would like to acknowledge the team of collaborators at the John Wayne, Dr. Richab Gupta, Dr. Dave Hoon, Dr. Guy Gammon, and those many others that have worked on this project over many years.

Thank you very much.

(Applause.)

DR. NOGUCHI: We're just a little bit ahead of time here, but I still would like you to try to get back at about 9:40 so that we'll try to keep on schedule.

Today and yesterday's speakers, at the back of the screen, we have some refreshments, and everyone else, at the same place as yesterday.

Let's be back at 9:40.

(Whereupon, the foregoing matter went off the record at 9;23 a.m. and went back on the record at 9:43 a.m.)

DR. NOGUCHI: Now, when we're talking about autologous and allogeneic tumor vaccines, most of the time we're talking about actually using tumors themselves or the putative antigens for them.

There are other parts of the body though that do react to that, and next talk before our panel discussion is going to be on autologous or allogeneic tumor derived heat shock protein-peptide complexes.

Now, this is something I know a little about because one of our scientists work on Josophela (phonetic) where heat shock protein is a major constituent, but I think it's going to be very

interesting to see how this fits into the whole tumor vaccine paradigm.

And to present this today will be Dr. Richard Young from MIT.

Dr. Young.

DR. YOUNG: Thank you, Dr. Noguchi, and thank you, Dr. Puri, for the opportunity to come and present this work to you.

Dr. Morton just reminded us of some of the lessons of history, and it reminded me of a lecture at MIT by a famous physicist last week who was much less polite in reminding us of a historical lesson. He said, "Many of you are too young to know this and the rest of you are too old to remember."

(Laughter.)

DR. YOUNG: What I'm going to do is to talk about something that is a bit more of a reductionist consequence. It's a consequence of a reductionist approach to what you've seen so far with autologous cell vaccines.

I'm, in fact, going to talk about a highly defined heat shock protein recombinant approach. This work focuses on -- I'm going to go through several topics. First, I'm going to describe some of the history that led to realize

that heat shock proteins have a specific utility for immunotherapy. I'll tell you a little bit about the design of these heat shock protein fusions. I'll describe some preclinical evidence for efficacy, and then I'll end by describing the manufacture of clinical grade material where the identity, purity, and reproducibility in the manufacturing process is quite critical.

The history of this actually begins in the early '80s when Douglas Young and I realized that the immune system in humans and in animal models during mycobacterial infection was focusing on a limited set of antigens, and when we identified these antigens, it turned out that they were classical heat shock proteins.

Now, quite a bit was known about heat shock proteins at this point, and we began to think it was possible that, in fact, not just in mycobacteria, but in many other bacterial, fungal, and parasitic infections that one would find that the immune system focuses much of its attention on these specific antigens.

In bacteria, the two major heat shock proteins are HSP-70 and HSP-60 or 65, and those two proteins can account for up to 20 percent of the

total protein mass in bacteria that have been stressed by infection.

So there are abundant antigens that are seen as among the immunodominant targets of both antibody and T cell responses. In fact, in mycobacterial infections in mice where it's been quantitated and appears to be similar in humans, about 20 percent of the entire CD-4 T cell response that is focused on mycobacterial antigens is devoted to HSP-60 and HSP-70.

So they're immunodominant antigens, and it's emerged that these proteins are in a class of proteins called molecular chaperons, and the job of molecular chaperons is, in fact, to facilitate the folding of proteins and to facilitate their unfolding an elimination from cells.

Moreover, we know a whole lot about these proteins. Not only do we know their sequences, but we know their crystal structure, and this is an example of just a piece of bacterial HSP-70. It's a substrate binding domain, the C terminal half of HSP-70.

So these are very highly characterized proteins. We know and understand them in many cases down to the three Angstrom level.

Why heat shock proteins for immunotherapy? Well, what I hope to show you is that, in fact, they're powerful immunostimulants. They can be used in an adjuvant independent fashion. Their action in all of the experiments I'm going to describe to you is occurring in the absence of any adjuvant. They elicit powerful humoral and cellular responses, and I'm going to show you some preclinical efficacy in tumor models.

We use fusion cassettes. We use either mycobacterium tuberculosis or mycobacterium bovus BCG HSP-70 and HSP-60. These, whether their origin is in tuberculosis or in bovus BCG, the sequences are identical.

And what we do is make these proteins as recombinant protein fusion so their covalent linkages -- these are single protein molecules then that will have attached to them a protein component of either an infectious pathogen or in several cases I'm going to talk about antigens potentially useful for cancer immunotherapy.

The cassette approach allows us to make recombinant fusion proteins single molecules that are easy to characterize. We have two choices in these cassettes. We can either make a recombinant

HSP-65 or recombinant HSP-70 fusion. We can choose any tumor associated antigen for which we have a DNA sequence.

It's a hybrid protein. It's administered, as I mentioned before, in an adjuvant free, saline formulation, and it elicits tumor antigen specific cytotoxic C lymphocytes.

The first model system I want to describe to you employed an HSP-70 ova fusion. This is a fragment of ova that represents immunoacids 161 through 276. In that fragment there is a very well studied SIINFEKL epitope for H2B, and we collaborated with Herman Eisen to study the ability of this fusion molecule to elicit CTLs and protect against B-16 melanomas.

The protocol we've used is to immunize mice, C-57 black mice, on day zero with a boost at day 14; to measure CTLs at day 24; and to challenge animals on day 24 and score tumor growth.

Here's an example of the data we've obtained. Where we take splenocytes from animals immunized with either the ova HSP-70 fusion, a control fusion protein containing HIV P-24 fused to the same fragment of ova, or that fragment of ova produced and administered on its own, and we've

examined two kinds of targets, either T2-K of B cells that have been pulsed with the SIINFEKL peptide or the same T2-K of B cells pulsed with an irrelevant peptide.

And what you can see is only in the case where we have splenocytes from animals that were immunized with the HSP-70 ova fusion do we, in fact, get significant cytolysis of this clone.

The response is quite avid. This is a peptide titration where we've used a cytotoxic T cell clone specific for SIINFEKL, and if compared, the titration in this cytolysis experiment exhibited by where we have a range of peptide concentrations used to load the target clone, and we've compared the ability of the CTL clone to lyse these targets relative to splenocytes from either the control ova albumin immunized mice or mice immunized with the ova HSP-70 fusion.

And remarkably, the half maximal lysis that you see across this titration is the same for this very avid CTL clone as it is for the splenocyte population in these animals.

These are CD-8 CTLs that are exhibiting this behavior. This is one of the experiments that demonstrates that. If we take both splenocytes and

stratify them according to whether they're CD-4 depleted, CD-8 depleted, or if we take the CD-8 enriched population, it's the CD-8 population that appears to be responsible for these immunological behaviors.

Now, when we've taken B-16 melanoma cells that have been derivatized, a cell line called MO-15 that's very well characterized that expresses ova albumen and we've asked what is the effect of taking mice that have been immunized at day 24, challenge them with approximately ten to the five tumor cells, what we've seen is that in control mice or in mice immunized with ova albumen alone, that there's very poor survival.

Where, in contrast, animals that have been immunized with the recombinant HSP ova protein and saline, in fact, show reasonable survival, and we followed these animals out there now for more than ten months, and they've exhibited this level of survival.

So we see protection by HSP-70 ova. We obtain ova specific cytotoxic T cells, their Class I restricted CD-8 cells. They recognize the specific epitope that is typically recognized by C-57 black mice when one immunizes with ova albumin and an

adjuvant, and I've shown you preclinical evidence that we can obtain prophylaxis against an ova melanoma challenge.

And we published this work last year. Now, what was striking to us is that the literature tells us that, in fact, if you take soluble ova albumin, you cannot elicit ova specific CTLs even up through a range of one milligram of ova albumin.

If, in fact, you derivatize this in some way to make it a particulate, you can, in fact, elicit CTLs, but we have a completely soluble antigen we're looking at.

So that suggests to us that something unusual is occurring that is a consequence of the HSP-70 protein being there. It turns out it is not a consequence of its presence per se, that is, mixtures of HSP-70 and ova albumin will not do it. It has to be a fusion.

And so we've come to wonder at the mechanism by which this occurs, the classic version of antigen presentation pathways are just summarized up here in which exogenous soluble antigen is typically endocytosed, brought into a lysosome where it's degraded. It's associated with Class II and

presented on the surface of cells in the context of Class II antigens.

In contrast, the antigens that end up going through the Class I antigen presentation pathway have typically been described as endogenous antigens, and the ability of these HSP-70 ova fusions to elicit as a soluble antigen a Class I restricted T cell response suggests to us that either there's some violation of this standard pathway and/or that the HSP-70 fusions are driving the antigen toward dendritic cells, which you heard yesterday have a capacity to present antigen obtained from outside cells via Class I pathway.

Now, I want to turn to some work that's been done primarily at Stresgen Biotechnologies in collaboration with us on an HSP-65 HPV E-7 molecule. The HPV is, as you know, the most prevalent viral sexually transmitted disease. It infects 30 to 50 percent of the sexually active population. The virus can be detected in greater than 90 percent of cervical carcinoma. HPV-16 is thought to be the most prevalent etiologic agent, and it's detected, as you know, by Pap smear.

The HPV associated cervical cancer, CIN I/II, is found annually in 300,000 to 1.5 million

individuals, CIN III in 65,000. Invasive cancer affects 14,000 or more individuals, and that leads to almost 5,000 deaths per annum all in the United States alone.

Now, we thought what we'd do is to make a fusion with the E-7 antigen. That's because the E-7 antigen is essential for transformation. It's expressed continuously as far as we can tell in infected cells. It's a true tumor specific antigen. It's not always clear that tumor associate antigens are, in fact, tumor specific.

It's well known to be a CTL target in humans and an abundant A-2 containing population, such as you see in North America. It's relevant that there are A-2 epitopes in the C-7 antigen.

So the fusion protein that we've made here is a fusion with HSP-65 from BCG. It contains the entire heat shock protein fused to the entire HPV E-7 protein.

So it's a single molecule. It can be purified then as a single recombinant protein.

The model we're using is a model for cervical carcinoma developed in T.C. Wu's laboratory. It's called the TC-1 model. It's developed here at Johns Hopkins. It's developed by

co-transforming cells with HPV-16 E-7, E-6, and Harvey-RAS.

And immunotherapy has been previously demonstrated with vaccine E-7 lab constructs.

The experimental protocol to examine tumor regression and rechallenge was to take C-57 black mice, to implant that TC-1 tumor on day zero. About ten to the five cells were used in this implantation, and on day seven we administered about 100 micrograms of the recombinant fusion protein with a boost at day 20 of a similar amount, inoculated to the scruff of the neck.

Tumor incidence is scored throughout. The subset of the animals that did not show tumors have been rechallenged on day 45, and we've continued to score tumor incidence.

And the data that we've obtained is shown on this slide. What we're scoring here is percent tumor incidence in various groups. There's been about nine mice per group, and what you can see is that animals that did not receive the fusion protein but rather received the saline control; in fact, ultimately obtained a tumor load that led to 100 percent incidence.

Whereas animals that, in fact, received the inoculation by day 35 were largely free of tumors. If we take the animals that are, in fact, completely free of tumors and rechallenge them now with a super tumorigenic dose, about five times ten to the five TC-1 cells, what we find is that for a transient period there is some tumor incidence, and those animals then recover and exhibit essentially no tumor load.

Whereas control animals that have been inoculated with saline and added to this part of the protocol in order to insure that, in fact, the tumor cells are still quite active and show that that control, in fact, works.

We wanted to know if instead of a boost, instead of a vaccination and a challenge we could do a single dose therapy, and so, in fact, this experiment was designed to do that where about ten to the five cells are implanted on day zero. We do a single treatment on day seven with the HSP-7 immunotherapeutic, and then we score tumor growth.

And the results are shown on this slide. What you're looking at is the percent tumor incidence in these animals and the days after injection of the TC-1 tumor cells, and what you see

is that in the control animals where they've been injected with saline, there's a very high tumor incidence. If we've injected another control, which is the E-7 molecule alone, there's a very similar very high tumor incidence.

Whereas if we have used the HSP-7 fusion protein for this therapy, then in fact, we can eliminate tumors from these animals.

So I've shown you that we can elicit protection in a therapeutic mode with these TC-1 cells and this model of cervical carcinoma. We can get subsequent protection from a rechallenge with a high dose of tumor cells.

I've also shown you that we can use a single dose treatment at a distal site. We see now long-term survival. These animals have survived for great than ten months, and we have Phase I trials with this reagent planned for the first quarter of next year.

I want to turn now to discuss just briefly the production of clinical material. We produce this material in E. coli. It's a standard E. coli culture. The HSP E-7 is in cells. It's release on lysis. The crude HSP E-7 is obtained by removing cell debris. That bulk product is purified

from the crude lysate by multi-columned chromatography processes, and ultimately the purified bulk product is diluted in the formulation buffer and filled into vials.

These are the specifications that we follow for any of the product. The bulk products release, if it meets standards relating to identity, which is a peptide map I'm going to describe in just a moment; the strength, which is the concentration of material; and the purity as measured by SDS-PAGE.

It's also tested for some specific impurities including endotoxin, the bioburden, and DNA contamination. Other characteristics of the product are also met, the appearance and pH and osmolarity, and in combination we think these tests tell us what it is, how pure it is, and by following the manufacturing SOP, we think we know how to do it again.

This is the peptide map that we can reproducibly obtain from the product, the HSP-65 E-7 protein product. It's produced by a proteolytic lysis of the product, and this HPLC profile is then assayed by mass. spec., and the mass. spec. gives us atomic resolution. We can identify each individual peptide. We can even identify peptides that are

partial digestion products, and in this way get a very high level of identity.

We can also examine the product by SDS-PAGE. Here you see two different preparations. This is under reducing conditions. The product by our analysis by scanning is at least 95 percent pure.

And I've told you that we have a well controlled manufacturing process. We have established rigorous quality control procedures. These include identity, purity, strength as measured by the concentration, and stability as assayed by, in a temporal fashion, the quality of that peptide map.

So where are we now with the E-7 immunotherapy? We have preclinical evidence for efficacy, a well controlled manufacturing process, and as I mentioned, our first clinical trial with this material is planned for the first quarter of next year.

Finally, I'd like to conclude by thanking my collaborators, Kimiko Suzue, and M.D./Ph.D. student at MIT and Harvard. Herman Eisen has played a critical role in the analysis of cytotoxic T cells. Hidde Ploegh at Harvard, who's

collaborated on much of this; Marv Siegel, who's Vice President for R&D at Stresgen Biotechnologies, and a very talented team of his composed of Lee Mizzen, Randy Chu, and Leslie Boux.

Thank you very much.

(Applause.)

DR. NOGUCHI: I'd like to now invite all of the speakers for this session to join us at the table and to supplement us, we're also going to have Dr. Michael Hanna, Dr. Marvin Siegel, Dr. Jeanne Novak, and Dr. Earl Dye.

And I think the way we'd like to do this part of it is to start to get some discussion going on the four questions that we've posed to you already. I'm going to just briefly read the outline of what we have and then ask the panelists who have not spoken yet to answer one or more of the questions. Because of time we would suggest that you pick one and try to address that in some detail.

When we're talking about products, and just to reassure everyone, while we are very happy that some folks can do peptide maps of the precision we just saw, we're not going to require that for everyone yet.

(Laughter.)

DR. NOGUCHI: But that's kind of where you ultimately want to go, but given that that's the gold standard, product characterization is going to be somewhat less than that, and some of the questions are what do you all think as a panel might be the most appropriate test.

We've already heard from several speakers that the concept and the ability to do a pre-immunization potency assay is somewhat problematic, and we do recognize that, but we would like some help on helping to figure out exactly how we can both assure the quality in terms of the product as far as potency goes and yet still be able to move forward in this field.

Purity is another question, and obviously if you can do a recombinant fusion protein and get -- I was kind of surprised there. That was only 95 percent pure. Actually for most of our recombinants we're shooting for a little bit higher purity, but for the rest of you all, I think that it'll be a little bit of a different concept of what purity is.

And then in terms of specifications, once again, I think the last presentation was sort of where we would like eventually people to be, but

short of that, what are the meaningful parameters that are going to help us in this?

Now, what I'd like to do is first call on Dr. Hanna to see if he has a specific topic that he would like to really address from those four questions.

DR. HANNA: I would like to take a few minutes to address the topic of potency and the post immunization value of delayed cutaneous type hypersensitivity in vaccines where it's important.

Now, for allogeneic vaccines, by nature these vaccines are going to be immunogenic. The question is: is it a functional or effective immunization?

For autologous vaccines, by nature they should not be immunogenic, except for tumor cells that may have a small proportion of tumor associated antigens, and in this case, this is what you hope to achieve with an autologous vaccine, is an effective immunization.

I have a few slides to make this point, and then we can open it to discussion. Could I have the first slide, please?

This is a study that was performed through the Eastern Cooperative Oncology Group, and

it was an autologous colon tumor cell vaccination program. In this study, induction or primary immunization with three vaccines, giving one a week for three weeks, was the regimen.

And when it was first reported, the intent to treat analysis was that there was no significant improvement in outcome in Stage II or III colon cancer patients based on immunization, but because we took a decentralized manufacturing approach, basically a home brew vaccine production approach, there was discovered that many patients did not get the treatment that they were supposed to get or the treatment didn't meet specifications.

So an evaluation was made of those patients that got the specified vaccines at the proper dose, and it showed a very strong trend towards improved outcome in the treated group versus the controlled group.

And then we took and went one step further, and the team of us, the ECOG investigators and myself that was exploring this data, then looked at those patients that did get immunized and looked at their delayed cutaneous hypersensitivity response to their third and final immunization, which was

autologous irradiated tumor cells alone, and compared that to clinical outcomes.

And you can see that for both survival and disease free survival, those patients that did not have a significant induration had a very poor outcome for both survival and disease free survival. In fact, it was not significantly different than the surgery only control group.

But in patients that had what would be considered by the Mann II test criteria a significant DTH, the outcome was much improved, and for patients that had greater than centimeter induration, it was even better, indicating that DCH could be a very important surrogate endpoint that takes into consideration both potency of the vaccine when it's administered, status of the patient's immune response, and tumor burden.

The reason I say this was these differences were significantly different in Stage II colon cancer, but not in Stage III colon cancer.

The next Phase III study we did where we kept an eye on this surrogate endpoint was a study where we gave the induction immunizations and boosted at six months, which this study indicated would have been helpful.

In this study, we had two indurations to measure, the induration to the third vaccine and the induration to the boost that was given six months later, and I show this slide just to show you that the basic tenet of immunology is that a boost should be equal to or greater in terms of reactivity than the primary immunization is true because you could see the fourth vaccinations, the DCH, were equal to or greater in most cases than the primary immunization, and the resultant outcome on an intent to treat analysis in all patients, Stages IIs and IIIs, was a significant difference in disease free interval, and even more importantly, a statistical difference in disease free survival in the Stage II patients and not the Stage III patients.

So it makes a point that the immunization could be effective and tumor burden could be the limiting factor.

Thank you very much.

DR. NOGUCHI: Mike, thank you for presenting that data.

Why don't we spend a few minutes here discussing it? Because I think that this represents one of the biggest dilemmas that FDA faces. Here we have some very impressive data in terms of a direct

test on a patient, but the real critical issue we would like to explore is what can you do before you actually inject such that you at least have some idea that is going to have a chance to have that DTH?

I know the people from AVACS have been struggling with this, but I'll open it up to the panel here to see if we have any further thoughts on this particular issue.

Yes, Mike.

DR. HANNA: Our major criteria for potency in both of these Phase III studies was viability and metabolically active irradiated tumor cells. We had to have greater than 70 percent viable cells going into the immunization.

DR. NOGUCHI: Is that good enough for the panel? Do you think that's going to be good enough or are there other things that might be done?

Early.

DR. DYE: I think that autologous vaccine certainly presents some very unique problems in terms of trying to assess their potential to do benefit in these patients, but I think that it --

DR. NOGUCHI: Yeah, Earl, put it right in your face just like FDA.

(Laughter.)

DR. DYE: I don't know if this mic is on. Can you hear me in the back?

Okay. I think that the point I'm trying to make is I agree very much with what the speakers have had to say here today in regard to the uniqueness of the autologous vaccine product situation. I mean, we're not faced with the advantage of being able to do a great deal of characterization on these kinds of products before they need to be administered to patients.

And so it behooves us during the early stages of product development to develop an understanding of what the important critical criteria are associated with these vaccines that do benefit in patients.

If assessments of viability or metabolic activity are important components that elicit responses in patients that can be measured and correlated with clinical benefit, then these are the kinds of things that need to be followed, monitored, controlled for in the development of these kinds of products.

DTH type reactivity may be a perfectly acceptable form of assessment of biological activity

of these products, but it needs to be demonstrated that there is some relationship between that type of a response in patients and the ultimate clinical benefit that they intend to derive.

The real challenge for us is to try to demonstrate that these vaccines are doing some benefit in patients. If we can identify correlates of clinical benefits and clearly establish that they do represent a measure to predict how these things are going to perform in patients, then that's what we have to try to do.

DR. NOGUCHI: Okay. I think that was an agreement. Is there -- yes, from the audience.

I feel like Jerry Springer up here. So --

(Laughter.)

DR. NOGUCHI: Yes, please use the microphone so we can all hear the question or the comment. Actually, we hope it will be advice.

DR. SOSMAN: I guess I had two comments and two questions, and the comments are really just to instigate not a riot but discussions.

And I guess the two comments are one is obviously very simple. You know, I guess my feeling as opposed to Dr. Morton's is that, you know, tumor

immunology has arrived because of all the great scientists that are in this audience, not attorneys, but that was just one.

And the other is I guess I see autologous tumors and allogeneic tumors differently than the panel. I see that this is an important field. I mean, I'm not interested in working in it, but I think it is a critical field.

(Laughter.)

DR. SOSMAN: No, no. I mean I certainly want to take the side that peptides are better, but maybe peptides aren't better. Maybe tumor cells are better. I agree.

But I don't quite understand where you go with this because it looks to me like allogeneic tumor cells and autologous tumor cells are really proof of principle, and that's what we should do with them and not then manufacture them for large clinical trials.

But then if we can prove a principle in small trials, then take them and develop products that are translatable to everything, there must be something in those tumor cells, and I agree. I thought, Dr. Morton, your talk was outstanding. I agreed with conceptually everything you said, but I

do have a question for you after this; that everything you said made sense.

But I would have taken from that is not to do the study you're doing, but instead try to figure out how to purify products that we could all use and we could all understand.

And those are the two, and maybe I won't ask my questions.

DR. NOGUCHI: Okay. Let me just address that in a general sense. I think that from our point of view from the FDA, the question is which way should we go. Should we purify? Should we do this? Shall we use crude lysates, whatever?

What our basic bottom line is we're looking for whatever works. Now, it's easier in a way to use well specified types of products because the control activities for that are quite straightforward, but that does not necessarily mean that they do or don't work any better.

And unfortunately, you know, it's sort of what will you approve? Whatever works.

Now, yes. Let's have Bernie take a crack, too.

DR. FOX: Sort of a comment to Jeff's comments. We do autologous tumor vaccination as

well as other model studies, but in the models if you vaccinate with an autologous tumor in the case of a melanoma in D-5, which is the same tumor that Jim uses, if you look in the adjoining lymph nodes of those animals, you find T cells that are specific for at least five of 20 GPU-100 peptides. They also recognize TRP-2, but don't recognize other peptides, any of the other 15 GPU-100 peptides or other ova peptides.

So as a comment, I'm glad Jeff's getting back up, but as a comment to that, I think that while peptides are nice, being as this is an autologous and allogeneic vaccine panel, that at least we know that at least in some models that vaccination with the tumor does give you specific peptide reactive T cells.

DR. NOGUCHI: Dr. Hanna.

DR. HANNA: I think that the peptides are nice, and Don had made a point to me that if we had had this meeting 15 years ago, there'd have been 12 people here and a few people wandering in and out and wondering if we're not on the fringe.

I think that when ten, 15 years passes and we have this meeting again, if we have the peptide data that shows clinical effectiveness, we

would all go in that direction, but clinical trials have to be conducted before a decision can be made as to which is the best way to go.

The autologous and allogeneic cell vaccines could be the control groups that the peptides would have to compete against.

DR. SOSMAN: No, I actually don't even think -- I mean, it may be right that you could isolate. There'd be too many as Bernie said, just too many to isolate, but then what you're going to really have to do is figure out how to make it simple because, you know, I treat patients like a number of people here, and it's just not feasible to do this.

And what you're going to have to do then -- I mean this is obviously my opinion -- is you're going to have to figure out a way to get it out of paraffin blocks because that you'll have on everybody, but you're not going to have fresh tumor on everybody.

DR. NOGUCHI: Okay. A question here. Now, let's try to direct it a little bit back toward potency if we can.

DR. BYSTRYN: Well, maybe I can make a comment regarding potency assays, and it's really

important to differentiate between assays which are looking at the ability of the vaccine to induce an immune response in people where, for example, DTH, I think, is a very simple and good assay, and assays that are going to enable you to measure the potency of the product hopefully before it goes into a patient.

The problem with assays which look at the ability of the vaccine to induce an immune response in people to evaluate potency is that now you have two variables that you are looking at. One is the potency of the vaccine itself, but the other is the ability of the patient to respond, and that is going to make it very difficult to interpret the data.

And, therefore, I think that in terms of trying to think about potency assay, you really want to think about assays that you can do in vitro, some kind of an assay of that type, using animals as a way to examine potency again, I think, as a fundamental flaw, which is that if you immunize an animal with a human product, you're going to get an immune response to that, and you're not going to know whether it's a response simply because you have

a xenogenic protein or because this protein happens to be immunogenic.

And at that point all you are really getting is an assay of identity, and you can get identity a lot more simply by simply probing the product directly with whatever you have to assay.

So I think that in terms of looking at potency, you really want to focus on in vitro assays that are going to examine some aspect of what you think is important from the strength.

DR. HANNA: You know, I didn't mean to make it an either/or contest. In the FDA today for the BCTG vaccine it requires a variety of in vitro assays. It requires a variety of assays in preclinical studies, and the last I knew, as of last year, it required a functional test in patients where you immunize with one lot and show that 90 percent of them converted in the Mann II test to PPD.

So we have a history of vaccines, and we shouldn't reinvent the wheel. DCH has been a primary measure of both the vaccine's quality and the patient's ability to recognize it and response immunologically, and I'm saying that there's a

precedent for it. We ought to not fail to recognize it in the tumor vaccine situation.

DR. NOGUCHI: Dr. Seaver.

DR. SEAVER: Yes. I think in this approach, and I'm talking mainly about autologous, not allogeneic, and probably things have been dated before, is either you pay the piper now or you pay it later, and that's what I'd like to suggest.

If you can well characterize, if there is a test that you can do on the patient beforehand to say it's going to work or not work, I'm sure all of us would agree to do that, but let's assume that that isn't the case, and we do have this heterogeneous response.

I think the issue comes up it's not to say that we can't do a potency assay whatsoever, throw up our hands in the air, because think of it from the patient's point of view. Because I've coached some people that have had cancer and we're trying to figure out which trial to go into, and that is if I have a therapy that's relatively nontoxic over standard therapy which is relatively toxic and is going to really affect my lifestyle, then I'm probably going to opt for the nontoxic therapy first.

But I would like to know whether it's working or not. So the ability to do something on the patient later, whether it be DTH, whether it be T cell, I don't care. To have a response within a month, to know whether you should do this, especially since many of these autologous therapies are multiple injections, and if you're going to go through the expense of multiple injections and want reimbursement for it, we'd better have some way of saying that it is working in lieu of having, quote, unquote, these potency assays.

DR. HANNA: And I agree, and we test them at three weeks. We have a three-week assay, at three weeks afterwards. Now, if the patient has no DTH, other interventions may be warranted.

DR. SEAVER: Right, and that's what I'm saying, is maybe one can formulate with the FDA a tradeoff strategy that in that case a post testing is part of it.

DR. HANNA: Exactly. Thank you.

DR. NOGUCHI: Okay. Yes. Let's have some more comments.

Go right ahead, Jeanne. Dr. Novak.

DR. NOVAK: Yes. I think with regards to the issue of potency, one of the things that I'd

like to come back to is some of the comments regarding whether or not you have assurance that your product is going to work once it's in the clinic.

Now, I just want to touch back on the point that that's why we're doing Phase III randomized trials, to get an assessment of whether this product is going to work, and I think certainly if one can look at a potency assay that would be an absolute predictor of outcome, that would be the gold standard. There's no doubt about it.

But I think I would also have to say we need to look back at a couple of other historical perspectives. That also should guide us and hopefully the regulatory agency about how we view potency.

I think we should consider that this assay is in place certainly to help guide and give us assurance that this product, in fact, is consistently manufactured, and can we always find an activity test for a product that is always going to give us an outcome or give us a handle on how it's going to work in the clinic, and I would tell you that based on preventive vaccine work, there are certainly a number of vaccines where the potency

assay is not always directly correlated to what one might expect in the clinic.

Many of those assays are oftentimes just an assessment of immunogenicity, and a lot of that work was done before some of the key antigens or protective epitopes, for example, had been identified, and it's only now as vaccines, for example, preventive vaccines, are moving towards well characterized technologies where one can begin to ask more rigorous questions because you have the tools and the ability to do that.

Again, it's not to say that we shouldn't be looking for that gold standard, certainly an activity test where you can have a high assurance of the activity in the clinic, but I think we need to come back and also think about are there activity tests or in cases of autologous vaccines where you are faced with a time line, are there other types of assays, analytical assays or characterization of the product that would give you an assurance that if your product is alive, for example, it's viable or if it expresses a particular antigen at a certain level or has other characteristics that you've found in previous studies to have had some correlation

with outcome, then those, again, are assays that I think should also be considered.

You can't discount that because, again, we need to go back to help FDA feel comfortable with how do you release a product and what do you set as a specification.

So I would also propose, in addition to continuing to look for the gold standard, to consider potency assessment as part of a total quality assurance package, certainly a validated part of an aspect of assuring validated manufacture, but also looking at parameters that could, in fact, provide some assurance based on the initial clinical data that you have, such as, again, correlative assay, be it analytical only rather than functional.

DR. NOGUCHI: Okay. Let me put this back to you directly then. Is cell number and viability and the correlate of a certain amount of DTH reactivity in the clinical trial, albeit after it has to be measured three weeks after the injection, is that enough? Do you think that's appropriate for this stage of development?

What other correlates can we really look at?

DR. NOVAK: Yes, I think that is a very good question, and I think I have to stay relatively open on that because one of my concerns always about, for example, the DTH assay, relying on something that's post treatment one has to say how do you make a decision about the manufacture or administration of that product when it's post treatment.

So the caveat there, I'd have to agree is that you're already into an administration process, but if that could be translated to developing a useful assay on the same premise so that that could be done at the time of manufacture or potentially gaining a particular history with that activity in a particular patient base, maybe there has to be room to look at that option for potency.

I think it's a very difficult question for the autologous vaccines.

DR. NOGUCHI: Okay, yes.

PARTICIPANT: I'd like to address this across the whole panel.

Since we're not sure what specific immune test to monitor, might it not be useful, and I'd be interested in the gamut of opinions, might it

not be useful to sequentially measure perhaps some nonspecific parameters, C reactive proteins, circulating IL-2R, neopterin, changes in TNB subsets, and then perhaps to try to use that, and that could even be done retrospectively as a marker that we gave this vaccine and something was happening physiologically, and then to go back and look at that in comparison with who responded and who didn't respond.

DR. NOGUCHI: Okay, panel. There's a potential way to address this.

Yes, Dr. Morton.

DR. MORTON: I think the problem is that the very key point that Jean-Claude Bystryn made is that the ability to induce a response will vary with the tumor burden, the stage of the patient, and in our work with the genetics of the patients.

These are self-antigens or modified self-antigens, and we know from work in animal models that immune responder genes are real, and so you'll have one patient that will respond to a particular antigen but not to another antigen, and vice versa.

And so since when you're trying to base the characterization of your product on the clinical

responses in the patient, it's not a valid -- in my estimation it's not valid because the response of the patient is going to depend on so many factors.

So I think in order to assure consistency in manufacture, you have to have some markers. You have to have some milestones that tell you you have, in fact, produced a consistent product from lot to lot.

DR. NOGUCHI: Well, I think the point being here is we know that response is going to vary per person, but are there some responses that are more nonspecific so that you could at least say this has some potency, and if the patient can respond, then at least I know that this lot of vaccine is worth injecting.

I think that's kind of what you're getting at. Is there something nonspecific we could measure that --

PARTICIPANT: Well, suppose we give a vaccine and it's going to elicit regression of a metastatic disease. Now, that has to reflected. In our state of the art, certain we wish we had better tools, but that's going to have to be reflected somehow in other kinds of intermediary and detectable markers.

And perhaps that might be an early detection of some kind of physiological activity even in those patients where whatever immune response was mounted wasn't effective in producing a clinically observable regression.

DR. NOGUCHI: Right. I think it's sort of like there still remains the two issues. One is the individual who has a variable response, but the other is trying to get something that has some consistency to it so that if the patient can respond they will.

DR. MULE: The whole premise in many of these immunization strategies is based on the concept of eliciting a specific response. That's the whole basis of many of these vaccines.

And Session IV actually is going to tackle a lot of the issues surrounding immunologic monitoring, and maybe some of these questions should be delayed until we hear more about appropriate immunologic monitoring in the next session.

DR. NOGUCHI: Any other comments? Yes.

DR. HANNA: Plus there's another point. We've had a lot of experience with in vitro assays. I mean this place here and the building behind it developed most of them.

What we learned from them is that on a population basis, they seem to correlate, but when you got down to the individual, you couldn't make a decision in terms of response or no response, but if you had an average, it seemed to correlate with responsiveness of a group, and that came from syngeneic mouse experiments.

DR. NOGUCHI: Bernie, did you have a comment?

Okay. Yes.

DR. LIVINGTON: I think the question is quite different for autologous and allogeneic vaccines. The allogeneic vaccines, as Don Morton has described or Jean-Claude Bystryn or others who are using allogeneic vaccines, readily are amenable to potency assays with antibodies, as has been described very nicely today.

The whole basis for autologous vaccines, which are so much more cumbersome to use and prepare is individually specific antigen, the mutated self-antigens which are, you know, so important in mouse models, and that, I think, inherently is impossible to determine in advance in the vaccine.

And so I guess you're thrown back even in the autologous to using some of the shared

antigens which have been defined to gauge potency, but I must say I have a sinking feeling about that because really your whole goal, if you want to immunize against these shared antigens, the allogeneic antigens or higher tech. vaccines, allogeneic vaccines or higher tech vaccines are a better way to go.

The only reason to go to the trouble of autologous is individually unique antigens, and I don't know how you gauge that. I think that's a pretty important question though.

DR. NOGUCHI: Okay. Yes, please.

DR. BYSTRYN: You know, taking into account the difficulty of measuring potency, I wonder whether -- and the need to, you know, move ahead with the development of products that may help the American public, I wonder whether at the present time maybe one possible solution will be to accept the suggestion that was made by one of the earlier other speakers that we talk about identity plus and that we think of potency perhaps as the ability to demonstrate in the vaccine the presence of a number of antigens that, you know, you believe may be biologically relevant, the assumption being that if the antigens were there, then the vaccine would have

the potency to induce an immune response to these antigens.

Because right now I think we're all having a very difficult time trying to come up with assays that can measure potency that don't rely to actually doing clinical trial with the vaccine to see what the immune response is that is induced, which is kind of, you know, potency post facto, which you probably don't want.

So my suggestion would be that we just look at potency, define it as the presence of relevant antigens in the vaccine, and of the demonstration of such antigens

DR. NOGUCHI: Okay. I want to use that comment sort of as the closing point of discussion for the panel. We've heard kind of all different sorts of proposals being sent around here, but let's just try to close with whether the panel thinks individually and as a whole is that going to be adequate enough.

And I want to just kind of close this by going right down from the end.

DR. SIEGEL: I think the issue that we keep talking about is really one of being able to predict or get a prognostic indicator that we are,

in fact, manufacturing the same thing over and over again. Granted that we don't know, as you said, on an individual patient basis whether they're going to respond, but at least can we control what we're putting in on a patient-by-patient basis so we can even determine whether or not there is any statistical correlation to any of those parameters?

I think we're forced to make a series of analytical measurements on whatever we're putting in, and in fact, can conduct those trials to see whether there is any correlation to maybe an antigen map or something like that that enables us to go back into the clinical trial situation and see whether anything correlates because at this point I don't know that there is a single entity or even a series of entities that you can use prospectively to say, "Well, if I have A, B, C, and D and not E and not F, then I will get a certain kind of activity."

I think you're almost forced to say, "Let me map what I have. Let me do the very expensive experiment instead of in mice, but in people to see if there is any correlation between what I put in and what I get out the other end."

Because I think that's where we are at this point. We don't know what to map.

DR. NOGUCHI: Dr. Young.

DR. YOUNG: I think I'd just like to echo a comment that Marvin just made, and that is to say I don't know how you deal with this entirely today, but I think very soon, in months if not weeks, you will have the ability to take this material and do genome-wide profiling and actually determine the precise number of messenger earning molecules for every species that you can detect on chips.

And although that is an expensive technology, you will be able to understand the identity of that material to an extraordinary level, and you'll be able to do cluster analysis on the information you get later on when you determine its clinical efficacy.

So I think it's a very difficult problem to deal with now, but in echoing Marvin's comments, I think understanding the material you're dealing with is just going to be critical.

DR. NOGUCHI: Dr. Novak.

DR. NOVAK: I think I'd like to just focus back on the issue from a regulatory perspective and with regards to the requirements for

having confidence in the release of a product based on purity, potency, et cetera, and ID.

The potency test, in my mind, for these products, again, as we've already discussed, is very difficult. If one holds to the strict sense of having an activity that correlates with the outcome, again, it's very difficult. We don't know enough about, especially in this case, the autologous vaccines because of the individual nature of these vaccines, and from a release point of view, it makes it very difficult when you have short time lines to do activity tests even in a generic sense, such as cytokine release assays or other in vitro assays that might give you a sense that there's some activity here, albeit it may not be directly related.

And I also agree with the comments that we certainly don't know all of the entities in these vaccines that are absolutely required for positive outcome.

But all of that said, I think that the challenge still has to be at this point in time because we don't have these advanced technologies, we don't what all of the antigens are that are

critical; we have to look at the parameters that we do know something about.

And we've heard today that for both the allogeneic and the autologous vaccines by ability is a factor, and I think that has to be quotiented into a total package where you can't necessarily look at an activity specifically and say, "Yes, this satisfies a requirement for release."

We need to look at the package in total, and again, I think that's a combination of antigen identification where possible, activities where possible and time permits, be it in vitro or in some sort of an animal model, and also as much characterization as possible, also keeping in mind that characterizing your product and setting up release assays is really just there are two different issues.

Your release assays are still only a subset of what you hopefully are doing as a total characterization of your product. So, again, we need to bring that back down to what we're talking about as far as product release and characterization and separating that from everything else you'd like to know about your product and hopefully we will

know as we continue Phase III studies and we continue further characterization.

DR. NOGUCHI: Early.

DR. DYE: Well, I think that pretty much sums it up. I think from a regulatory perspective Dr. Novak has really captured very eloquently the issues that face us with all biologic products, not only the autologous or allogeneic tumor cell vaccines.

I think it's critical to realize that there's a need to know from lot to lot or from patient to patient that the process that's being used to prepare these vaccines is preparing products that are going to do benefit to these patients and not cause harm.

It's important to know that if these patients are going to receive multiple injections of these vaccines, that the injections they receive the first time are going to be comparable with the injections that they receive at later times, and so there needs to be a way of assessing these products for the important characteristics that are going to define whether or not they induce the kind of response in patients that we're hoping to induce.

I think identity plus is a no brainer. Certainly we need to know what the critical components or markers are on these products that are going into patients, how they relate to the kinds of responses that we're trying to engender, whether or not things such as cell viability, metabolic activity, the ability to induce some sort of a functional response in tissue culture in animals or in patients are all part of a package that need to be assessed in terms of evaluating whether these products are going to be useful or not.

And I think that it's a challenge that we can't ignore, that we have to continue to look for solutions.

DR. NOGUCHI: Okay. In the interest of time, since all of the rest of you have actually had a chance to speak except for Bernie, but you're going to speak later, if there's any disagreement with what we've been hearing, this is your opportunity.

Yes, Dr. Mule.

DR. MULE: With respect to DC based vaccines, we discussed yesterday actually the complications involved in defining potency at this early stage. What complicates it, of course, is the

fact that we don't know yet, and you hear from Jacques Banchereau and Ralph Steinman that we don't know yet what dendritic cell to use.

We don't know how best to expose antigen to dendritic cells. We don't know how to best give dendritic cells. There's a lot of information about IV route of administration, interdermal, subcu. We don't know how long the vaccine is efficacious once it's injected.

So to me it's like trying to get to the ball before Cinderella's stepsisters. I think it's a little bit early to make clear-cut definitions of potency with respect to at least the DC based vaccines.

DR. NOGUCHI: Okay. What I'd like to do is just thank the panel and thank the audience for providing an extremely valuable discussion. I can assure you from our FDA perspective, we will be immediately looking and trying to evaluate this and spread the wisdom throughout all of our evaluations of all the vaccines, including the ones you can characterize.

So thank you very much.

(Applause.)

DR. NOGUCHI: Now we'll be moving right into our next session. One of the co-hosts here is actually my boss, Dr. Jay Siegel. Dr. Siegel has had a long and distinguished career here at the FDA and the NIH campus and is an immunologist of some repute. I think just a few years ago he reluctantly gave up the lab, but he certainly has not given up his interest in immune responses, and especially of tumors.

His co-host here will be Dr. Mario Sznol, who is the head of the Biologics Evaluation Section at the Investigational Drug Branch, Cancer Therapy Evaluation Program, NCI. We like those long acronyms, but Dr. Siegel will be opening this session.

DR. SIEGEL: Well, thank you.

Okay. I have an announcement that the poster abstracts must be removed from the boards no later than 2:30 this afternoon.

We now move to Session IV entitled "Preclinical Strategies and Immunological Assessment in Early Clinical Trials of tumor Vaccines." In so doing, we cross a bridge that several have ventured across already, moving from how to characterize the

product to how to characterize the immune response to the product.

I think this is a very exciting and important question. As we heard from Dr. Keegan yesterday, the immune response is not a measure of benefit, nor is it an accepted surrogate, and I would add that it's far from being one. We're several controlled clinical trials short for any given product in response and benefit of knowing, understanding a relationship.

Yet it's extremely important, as some speakers have noted, to select which strategies to go into clinical trials, to optimize the strategy, dose, and regimen, and the like, and I would add to that even after the demonstration of efficacy for a given product in disease, it will remain quite important as these sorts of products, vaccines and cellular products in general can be modified and improved in how they're made. They can be modified and extended in how they are used.

And in order that not every modification requires randomized controlled clinical trials, understanding correlates of efficacy will be critical.

To reach these ends then, it's critically important early in development to identify relevant immune effect or arms that are relevant to the intended mechanism of action and thus likely to correlate with or predict benefit, and to identify immune response measures which can characterize that effect or arm, and which can be performed reliably and reproducibly across time in a patient, across patients in a center, and importantly also, across sites in multi-center trials which are likely to be necessary.

So with that, emphasizing, I think, the critical importance of these topics, it's my pleasure to introduce our first speaker, Dr. Steven Rosenberg of the Cancer Institute and needs little introduction, who will talk about his work in identifying cancer regression antigens and using strategies to target those in tumor vaccines.

DR. ROSENBERG: Thank you.

In developing cancer vaccines, we need to understand two basic phenomenon. First, what is it we want to immunize against? And, secondly, what is the optimal way to perform those immunizations?

And I'd like in the next few moments to talk about our studies, trying to develop at least

the beginnings of answers to those two questions. What should we be immunizing against in the cancer patient, and how can we best perform those immunizations?

In attempting to find the antigens in patients with melanoma, a human tumor that we've used as a model, although as you'll see, we're beginning to extend beyond that diagnosis, we attempted to define the relevant tumor rejection antigens in patients by identifying the genes that encoded what we thought to be the relevant tumor antigens.

And those studies were derived from a pilot clinical trial performed here in the clinical center.

Can I have the first slide, please?

This clinical trial used a kind of cell we had defined in animal models and in humans called tumor infiltrating lymphocyte, cells that we could derive from tumors that in vitro exhibited specific antitumor activity and recognition of tumor antigens.

In a trial we administered them to 73 patients with metastatic melanoma. About a third of those patients would respond. This was about twice

the response rate seen with Interleukin-2 alone, and when these cells were administered with Interleukin-2 to patients who had previously not responded to Interleukin 2 therapy, again, about a third of patients responded.

And what this clinical trial did is provide us with a cell that was recognizing antigens that when administered to patients, adoptively transferred, were capable of mediating tumor regression.

And the question we then asked with the specific subpopulation of TIL cells that were involved in tumor regression, the question we asked was: what was the antigens, what was the chemical nature of the antigens recognized by these particular lymphocytes?

And the strategy that we utilized in these studies had four parts:

First, to grow tumor infiltrating lymphocytes from patients with cancer and identify the TIL cells that could recognize appropriate antigens in vitro;

To administer those TIL to patients, as I've just mentioned, and identify the selected subpopulations that could mediate tumor regression;

And then utilize those TIL associated with in vivo effects to clone the genes that encoded the antigens they recognized.

Now, it was necessary, of course, to then close the loop by utilizing those genes that encoded these putative cancer aggression antigens by evaluating clinical responses in patients after the adoptive transfer of lymphocytes sensitized in vitro specifically to those antigens or to utilize those genes or gene products in the development of cancer vaccines to see if, in fact, those selective immune responses could translate into tumor regression in patients.

Well, in beginning those efforts, we began with a patient shown here who had multiple tumor nodules. He received his TIL, along with IL-2, and showed a dramatic regression not only of these tumor nodules, but also intraperitoneal tumor as well.

This was patient 1200, and Dr. Utaka Kawakani asked what were the antigens that were recognized by this TIL that resulted in this tumor regression, and these were the first examples of our efforts in this direction.

I won't go into it in much detail because it's been published, but in these first series of experiments, two antigens, GP-100 and MART-1, standing for melanoma antigen recognized by T cells, were identified by TIL associated with tumor regression.

The GP-100 molecule, previously known as a molecule recognized by a monoclonal antibody, HMB-45, but unknown as a T cell antigen; MART-1 previously unknown in any gene or protein data bank, but the surprising observation was that both of these proteins were normal, nonmutated proteins present in melanocytes and melanoma cells, and in fact, the Northern Blot studies that were performed demonstrated the expression of these proteins only in melanomas, some in retina, no other normal tissues with the exception of melanocytes.

As we began further to define the nature of these reactivities, some additional surprising findings revealed themselves of 29 HLA-A2 restricted TIL that recognized shared melanoma antigens from patients, and this represents over 50 percent of all HLA-A2 TIL.

Twenty-one of these 29 that recognized specific melanoma antigens recognized the MART-1

antigen that we defined, and all of these 21 TIL recognized the exact same nine amino acid peptide, this AAGIGILTV, and no other peptide in this molecule.

Thirteen of the TIL recognized GP-100, five different epitopes. We've now actually identified an additional five, and they were heterogeneous. Eight reacted with both of these.

We have two TIL that have recognized tyrosinase, and those represent the three antigens recognized by all of the TIL that we've identified from melanoma patients.

It, therefore, appeared that many melanoma antigens were normal, nonmutated self-proteins presented on the surface of melanoma cells in normal melanocytes, and somehow the growth of the melanoma resulted in break of tolerance to these normal differentiation proteins because, of course, the TIL that were used to identify them came from the growing tumors of patients.

Now, this explains something which had mystified us for the previous ten years of our immunotherapy experience as exemplified by this patient, who is one of the patients who had multiple

melanoma deposits following resection of a primary lesion.

He underwent a complete regression of these deposits. That's just some melanin staining in the skin, and he showed us as the melanoma deposits were disappearing this vitiligo depigmentation, which on biopsy showed complete destruction of melanocytes in this area.

This then led us back to our immunotherapy clinic to look prospectively at all patients that were seen in our clinic at least one year after receiving Interleukin-2, and in none of 104 patients did we see, with renal cell cancer, did we see vitiligo. We saw it in 12 of 73 melanoma patients, again suggesting that somehow the growth of the melanoma had sensitized the patients to reactivity against these differentiation antigens that led to the vitiligo.

But more compellingly, if we looked at the melanoma patients, all of the vitiligo occurred in those patients showing objective clinical responses, either complete or partial regressions, and no vitiligo seen in nonresponding patients, providing what I think is compelling circumstantial evidence that it is the reactivity against the

differentiation antigens, the same antigens that are causing the response to the melanoma, that are also resulting in vitiligo.

Now, this leads to a conjecture which could potentially lead us to extend these observations to other tumors, and we're very vigorously pursuing this area.

If normal tissue specific differentiation proteins from melanocytes expressed on tumors can serve as tumor antigens, well, virtually every organ in the body contains unique proteins unique to that organ. Perhaps tissue specific proteins in tumors derived from other nonessential organs could serve as immunotherapy targets. After all, the loss of the epithelial cells of organs, such as the thyroid, the ovary, the testes, the breast, and the prostate, would be a very small price to pay for the destruction of the tumors that arose from those organs and continued to express those differentiation proteins.

Well, that was only part of the story. I'd like to present just two additional examples that demonstrate not only additional tumor antigens, but other biologic principles involved in how tumors present antigens to the immune system.

A 26 year old woman who had dozens of cutaneous metastases, melanoma in both tonsils, soft palate, lung underwent complete regression of her melanoma when treated with TIL cells and IL-2, and when Paul Robbins studied TIL-888, TIL associated with the complete regression in that patient, and another TIL, TIL-1290 that was derived from another lesion in that patient, he identified the beta ketenin (phonetic) molecule as the gene that was encoding the protein recognized by this TIL.

The base and amino acid sequence, however, revealed a single C to T mutation which resulted in a serine to phenylalanine mutation switch that resulted in a nine amino acid peptide ending in this phenylalanine that accounted for all of the reactivity of this TIL.

And so here's a case where a mutation in a normal protein resulted in the generation of a tumor antigen, and when Dr. Robbins looked at the normal sequence compared to the mutated sequence, there was a one million-fold difference in recognition by TIL from this patient.

Beta catenin, of course, a protein that reacts with the APC tumor suppressor gene product

and is quite important probably in the malignant phenotype of that patient.

And so a second principle of the degeneration of tumor antigens in patients is not only differentiation antigens, but mutation of normal cell products.

Well, the final example I'll discuss is this patient 586, who studied by Dr. Ron Fu Wong, who underwent a partial regression when receiving those TIL plus IL-2. Dr. Wong identified the gene sequence of the protein recognized by this TIL.

It turned out to be a protein TRP-1, another differentiation protein, but quite surprisingly, none of the peptides from the normal protein conferred reactivity to TIL 586. It was only when Dr. Wong then explored the third open reading frame that we found a 21 amino acid, probably nonsense polypeptide, encoded by the third open reading frame. So this is now an epitope coming from not the protein encoded by the normal gene, but by that same gene sequence, and it was the first nine amino acids from this 21 amino acid polypeptide that conferred the reactivity to TIL 586 starting in this methionine.

Now, clones of TIL 586 identified TRP-2, another differentiation protein as a protein recognized by this heterogeneous TIL in this patient that underwent a good partial regression, a second antigen.

But a third clone from this patient, all derived from the same TIL population, demonstrated a clone that now reacted with that patient's melanoma restricted by HLA-A31, not A31 negative melanomas, but now for the first time this TIL could recognize HLA-A31 breast cancers, but not normal cells from that same patient.

And so this patient was developing reactivity not only against melanoma antigens, but antigens now shared more broadly on other tumors, and when Dr. Wong identified this gene, it encoded the NYESO-1 as a gene product, at that point known only to be reactive with antibody, but not with T cells, as the antigen recognized by this patient's TIL.

And, in fact, two different epitopes on the ESO antigen were recognized by two different clones.

Interestingly, this antigen is expressed in about 25 percent of breast cancers, prostate

cancers, even more non-small cell lung cancers, and the 10-amino acid epitope was identified as well as a second epitope recognized by another clone in an alternative open reading frame.

And so we have to look not only at the known protein products of the genes that encode tumor antigens, but also their alternative open reading frames, as well.

And so as we summarize this patient, this patient's TIL, isolated from a growing tumor of that individual, recognized three different antigens, TRP-1, TRP-2, and two epitopes of the ESO antigen, as well. Most patients with melanoma are probably recognizing not only a single, but perhaps even multiple antigens, and we have several examples of this in our own patients.

Well, we've now described eight different antigens. I won't go into others, and there are others that are being found in the laboratory that recognize not only differentiation antigens, intronic sequences, mutations, alternative open reading frames, as well as some that are shared on other melanomas, and there are other antigens to be discovered as well.

It's quite clear that if you have a T cell that recognizes an antigen, you can clone that gene, and in this summary of 135 different TIL restricted by HLA-A1, 2, 3, 24 and 31, there are still TIL that we have that have antigens that are not encompassed by any of the genes and gene products we know about, and these are being cloned, in fact, a new antigen just found by Dr. Mammero Hirata in the last week, and so finding of these Class I restricted antigens is something which is vigorously ongoing.

Now, we've talked only about melanoma, and in fact, for tumors other than melanoma, it's very hard to raise Class I restricted CTL that recognize tumor antigens. However, one can generate from about ten percent of breast cancer patients CD-4 positive TIL that recognize tumor antigens uniquely, and this was published by Dr. Schwartzentruber and Dr. Topalian.

TIL cells from a breast cancer patient recognizing that breast cancer, but not normal cells from that patients or other tumors, and Dr. Dadmars along with Dr. Schwartzentruber have described from about a quarter of ovarian cancer patients CD-4 positive TIL that recognize unique antigens.

As you can see here, the autologous tumor being recognized in each of these experiments, but not autologous normal tissues or other tumors.

But the problem of recognizing the genes encoded by CD-4 cells is a far more challenging problem and one that up until very recently we've had no method for identification, and the reason for the difficulty comes from an understanding of how antigens are processed.

Antigens recognized on Class I by CD-8 positive cells are the result of the processing of intracellular proteins, which are cleaved and transported through the ER to the surface of the cell. Whereas Class II recognized antigens are exogenous antigens, in general brought into the cell, into different subcellular compartments, endosomes, that are then attached to Class II molecules and brought to the surface.

And if we try to use the classic expression cloning techniques that we've used, one cannot just simply introduce a gene into the cell and expect it to enter the Class II pathway. Somehow methods have to be developed to bring these endogenous proteins specifically into the Class II pathway.

And in the past several months work, again, by Dr. Ron Fu Wong has generated a general method for the cloning of genes expressed by Class II antigens by developing CDNA libraries with the vector encoding and variant chain sequences which target these transfected genes to the Class II pathway and doing that in 293 cells that are highly transfectable, that have been engineered to express the appropriate Class II DMA, DMB, and in varying chain molecules that are necessary for gene cloning.

And so utilizing now this new technique just being submitted for publication as we speak, using CD-4 positive cells that recognize a unique melanoma antigen restricted by HLADR, the gene cloning techniques were used, as I've just mentioned, by screening CDNA libraries, and this now first cloning technique identified a gene, a quite unique gene, recognized by this TIL, restricted by Class II, which is a fusion product of the LDL receptor gene on Chromosome 19 with a fructose transferase gene on that same chromosome, and the peptide epitope has been identified as well.

In other words, the gene for the LDL receptor, the gene for the fructose transferase as a result of a chromosome inversion gives rise to a

fusion product, probably a recombination between the two ends of this chromosome that gave rise to the gene recognized by this TIL.

A study of the individual amino acid epitopes has also identified the particular peptide epitope recognized, which is in this fusion product of the ligand binding repeats of the LDL receptor, as well a the fructose transferase gene, now as a result of a chromosomal rearrangement being read in the reverse direction, a nonsense sequence that gives rise to the peptide epitope.

And I've mentioned this just to illustrate again we know there are so many chromosomal abnormalities and mutations that occur in tumor cells that have an opportunity to give rise to mutations. This is the first antigen recognized by this approach. Dr. Wong has now identified a second T cell antigen and the epitope derived from the CDC-27 gene, and my suspicion is now we'll be able, using this general technique, to identify CD-4 restricted antigens in a variety of tumors as well.

Well, we understand a lot about the molecular nature of these antigens, but of course, the goal of these studies is to use them to develop

therapeutic approaches to these treatments to turn this theory into performance.

And as we engage in this effort, there are, of course, two main issues to deal with. One, the passive transfers, we've talked about passive immunotherapy or active immunotherapy, the development of cancer vaccines.

A daunting problem as we attempt to translate this into human trials, the number of possibilities are staggering. We have multiple antigens, multiple ways to immunize with peptide, protein, DNA, a variety of viruses, multiple adjuvants, routes of administration, and obviously very careful selections have to be made.

Based on animal studies, Dr. Nicholas Restifo in the Surgery Branch and his group have, over the years performed extensive analyses attempting to determine the general principles in animal models to use for human vaccination. I won't present any of his data, except to present the principles that we've used to try to determine how we approach this.

In general, based on animal models, immunizations most effective in generating reactive T cells and most therapeutically effective as we

looked at multiple cytokines, IL-2 and IL-12 turned out to be the most effective analogues to use with immunization.

The more immunogen we give, the better. Extending the length between immunizations seems to be important, as well as repeated boost immunizations with different vehicles containing the same antigen to avoid immunization to the vehicle itself.

Now, we've now treated over 280 patients with these different approaches, using adenovirus that encodes MART-1 or GP-100, supplied to us by the Genzime Corporation, very close collaborations with the Thereon Corporation, providing to us a GMP virus, vaccinian fowl pox virus encoding these genes, and studies with these products are very much ongoing.

I'd like, however, in the remaining few minutes to talk about our peptide studies because as of right now, these appears to be the best ways to immunize humans against these gene products, although the viral studies are very actively being pursued.

One of the problems we believe we have in some of these viral studies that attempt to use

viruses for immunogens is the problem with the fact that most humans have neutralizing antibodies against adenovirus, and we need to find ways to overcome this.

We're now looking at ways to use adenovirus to infect dendritic cells. Similarly, with pox viruses, patients have high titers of antibody against vaccinia, but not against fowl pox, and so we're emphasizing in our current study the intravenous administration of very high levels of anti-fowl pox antibody to immunize patients in these studies.

The most successful immunizations that we've achieved thus far, however, have been in patients receiving peptides, the immunodominant peptides from these proteins, and, in general, our best results have been obtained when we use peptides that have amino acid modifications that improve the binding of these peptides to HLA molecules.

Each of the immunodominant peptides we identified is a relatively poor binder, an intermediate binder to HLA-A2 for the antigens that are restricted by A2, and Dr. Miriah Parkhurst, by looking at hundreds of different modifications, has identified peptides with specific amino acid

modifications at the anchor residues of the peptides that can increase binding ten to 50-fold.

We've tended to concentrate on the GP-100 molecule, and in studies in which we've immunized patients with a variety of different peptides, a nine amino acid peptide beginning at amino acid position 209 that contains a methionine substitution that increase binding to HLA-A2.

And when we perform those immunizations, we can in virtually every patient get a strong reactivity not only against the peptide, but against HLA-A2 positive tumors, and an example of one such assay is the following.

If we immunize with the 209-2M peptide and incomplete Freund's adjuvant every three weeks with two immunizations and now just take PBMC from patients, mix with peptide, and seven to 12 days later simply look for reactivity against the peptide or tumor, we do not see it in patients prior to immunization that are sensitized with 2M, exposed to 2M peptide in vitro, but now tested against the native peptide. No reactivity, no reactivity based on gamma interferon release against tumors.

The patient is highly immunocompetent, can react to flu, but after two in vivo

immunizations, when we now look at the simple ten-day assay in vitro, we get very high reactivity against the immunizing peptide that also translates into very high reactivity against the A2 positive tumors, but not A2 negative tumors.

When we immunized eight patients with the unmodified peptide, only two showed evidence of weak immunization. When we used the modified peptide, and I won't go into more detail because we've just recently published this a few months ago, ten of 11 patients showed strong reactivity to the immunizing peptide, as well as to tumor. And we have, therefore, concentrated our efforts on these modified peptides as cancer vaccines.

One can by a whole variety of assays, ELISPOT assays, limiting dilution assays, demonstrate this immunization as well. We can never detect by limiting dilution reactivity at the limits of the assay, one in 30,000 frequency immune T cells against peptide or tumor prior to immunization. However, post immunization reactivities are in the one to three to 6,000 range. This would be the same precursor frequency that one would have after clearing the body of a natural flu infection.

And so it is possible to highly immunize patients. The problem is the in the face of all of these circulating precursors, we saw no true objective responses. We saw individual tumors disappear, but no patient that showed the strict criteria of an objective response until we then added Interleukin-2 to those individual peptide immunizations, and then in our pilot trial of 31 patients, 42 percent showed an objective regression compared to the 15 percent or so that we normally see with IL-2 alone.

We saw no increased activity when we gave these peptides with IL-12 or GM-CSF.

There's not a randomized trial, but if we look at 182 patients that we treated with IL-2, our response rate was 15 percent. If we look at patients who are simultaneously being treated with recombinant virus along with the same IL-2 regimen, 12 percent. This 42 percent appears to be up to three times higher as a result of the 2M peptide vaccination, but this requires a randomized trial to see if this is, in fact, correct.

The Cytokine Working Group is looking at 209-2M in conjunction with IL-2 to treat patients, and a Surgery Branch fellow extramural trial being

run by Dr. Schwartzentruber should be initiated soon, which will compare Interleukin-2 treatment to Interleukin-2 treatment with this peptide to see if, in fact, that provides effectiveness.

I'd like to show just some anecdotes, realizing they are anecdotes, that demonstrate some important principles of this treatment.

Patients that were treated in this trial showed with peptide alone, showed inflammatory areas around their subcutaneous deposits and many disappeared, but some would appear at the same time, and we saw no objective responses to that.

Patients that did have responses to the 2M peptide plus IL-2, such as this patient who had hundreds, if not thousands, of lesions, including ones growing out of her cornea, had a complete regression, and as these lesions disappeared, so too were destroyed the normal melanocytes surrounding these lesions.

I'll finish in another minute and a half.

(Laughter.)

DR. ROSENBERG: For the first time we saw lesions in the brain disappear, something we had

never seen with Interleukin-2 alone, as we see with these two lesions showing this shrinkage, as well.

And in this patient, this brain metastasis has disappeared completely.

I would close by just mentioning the trial we now have ongoing, and that is instead of using a single peptide for immunization, we're immunizing with four different peptides, two from the GP-100 molecule, one from MART-1 and one from tyrosinase, and now for the first time in our first 16 patients treated with these four peptides in the absence of IL-2, we're seeing responses to peptide alone, such as in this patient now who's had a quite extraordinary response of these lesions to peptide alone in the absence of IL-2. You can see disappearance of these, as well as in the posterior thigh.

And in this final patient I'll show receiving peptide alone who had lung lesion go away; liver lesions disappear with these four peptides; and intraperitoneal lesion as well as this large intramuscular lesion in the thigh. This patient went on to a complete response and then developed vitiligo as these lesions were disappearing with this peptide immunization.

Can I have the lights on please?

And so we're continuing our studies attempting to immunize not only against peptide, but against viruses encoding specific molecules that hopefully can be developed as successful targets for immunization.

Well, thank you for your very kind attention.

(Applause.)

DR. SIEGEL: Thank you.

There's been a minor change in program. The next two speakers have switched positions. So our next speaker and last speaker before lunch break -- is that correct? -- is Dr. Jeffrey Weber of the University of Southern California and the Norris Comprehensive Cancer Center speaking about his experience with immune responses to peptide pulsed vaccines.

Thank you, Dr. Weber.

DR. WEBER: Boy, talk about a tough act to follow.

(Laughter.)

DR. WEBER: Based on the immune tour de force that Steve talked about, as well as very eloquent data generated by Cass Malief and Tiery

Boone, I'm going to talk to you a little bit about some peptide trials that I've done at USC-Norris.

And if we can have the first slide.

I'm going to talk to you about tumor antigen peptide based therapy both in melanoma and in HPV induced preneoplasia, and basically there are a lot of good and bad things about peptides, some of which Steve has already alluded to.

The good news is that peptide vaccines for cancer, well, they're not toxic. They're cheap. Clearly he's shown and others have shown that they can induce immune and clinical responses.

The bad news is that not all patients have the correct haplotype if you have a single or even several peptides. Clinical responses in patients with metastatic disease, mostly melanoma, are uncommon. The ugly news is that there's clear evidence that there is immunoselection that occurs in vivo, and that can cause resistance, and Cass Malief and Martin Cast have shown that some peptides can even be tolerogenic.

The hope is that multiple peptides with potent adjuvants and potent cytokines will be effective.

As we've heard, MART-1, GP-100, and tyrosinase are all found in melanomas, and they are neoantigens. They are normal antigens found on melanocytes.

You can induce immune responses to these normal antigens in patients with metastatic or resected disease. MART-1 specific T cells are prominently found in blood or in tumor infiltrating lymphocytes, and as Steve Rosenberg implied, GP-100 TIL have been found to be therapeutical.

What we did at USC-Norris was to take the MART-1-27 to 35 nonomer, that peptide with incomplete Freund's adjuvant, and we took the nonomer which has been found to be an immunogenic peptide in vitro in work done by a number of investigators. It's also been found to be immunogenic in vivo. It's been found to be well tolerated.

And the question that we asked was: will patients with resected Stage III and IV melanoma at a high risk of relapse mount an immune response to MART-1, and more importantly, will the immune response correlate with time to relapse as a clinical endpoint?

The schema was very straightforward. It as a Phase I trial with cohorts of three or more patients who got 300, 1,000, or 2,000 micrograms of the peptide with incomplete Freund's adjuvants.

We gave them four injections three weeks apart subcutaneously, and the objectives of this typical Phase I trial were toxicity and did we generate immune responses.

We also skin tested with peptides. We did leukophoreses on all the patients to collect their peripheral bloods, and that was done prior to and after the series of vaccinations.

This was not toxic, as one might expect. We saw very transient, non-therapy related thrombocytopenia in one patient. The same patient also had a low white count. These were trivial toxicities. The vast majority of patients had some local pain and granuloma formation, but both the investigator, i.e., me, and the patients agreed that this was therapy that was well tolerated.

In this slide, which is probably a little difficult to read at a distance, it shows the immune assays that we did, and these immune assays are based on multiple restimulations of the patient's peripheral blood mononuclear cells three

times, done three weeks in a row, and then a cytokine release assay using gamma interferon ELISA as the readout was used as the immune response indicator.

And it shows pre and post, pre and post in order for 22 patients that we had phoresis samples on. We treated 25 patients. Three of them could not be phoresed due to access problems, and it shows in yellow -- and this just shows T-2 cells that are not pulsed with peptide; T-2 targets that are pulsed with MART-1; or 624 MEL cells that express the MART-1 antigen and are HLA-A2 positive.

It shows that you get boosting that is an increase of at least 100 picagrams per mL of gamma interferon post compared to pre in ten, if you can count them in yellow, out of the 25 or out of these 22 patients.

What we also did was to do cross-specificity assays, which I didn't show on that slide. It would have been much too complicated, and what we did is we would take the samples of blood from these patients after vaccination, and we would stimulate them with either the flu matrix peptide, which virtually all of them should respond to, or with the MART peptide, and then we would crisscross

them and ask whether there was reactivity by the flu stimulated cells to MART, which there should not be, and by the MART stimulated cells to flu, which there should not be, but the flu stimulated cells should show a response pre and post. Hopefully the MART should show an augmentation.

And in this patient, who was an immune responder, there's no question that in the cross-hatched areas there was a flu specific response pre and post. In the flu stimulated cells there was a trivial MART response. Pre, there was no MART response against MART stimulated cells, but a very nice response here post vaccination.

In a patient who was not a responder, nice looking flu reactivity, suggesting that the patient was immune competent against flu, but no evidence of any reactivity against MART, suggesting that the patient was a nonresponder.

And these are the kinds of assays we've performed for all of the so-called responders and for most of the nonresponders.

In addition, in patients who have cytokine release also have chromium release, again, much higher backgrounds. This just shows post MART

specific cytolysis. This is the post control, pre, pre. So there was some augmentation here.

Another patient who was a responder by cytokine release had post MART-1 vaccination, increased cytolysis here compared with control, and again, this is pre compared with control. So if you subtract this from this, obviously there's almost no background, and there's some significant activity, although with a background against a nonspecific target, post.

So those are the kinds of data we generated, and the question was: was there any evidence in this very small trial of only 25 patients of whom 22 actually had assays available; was there any evidence that there was clinical benefit that correlated with the immune response indicator?

The median follow-up is 16 months. Nine of the 25 patients who had Stage III and IV disease have relapsed. Three have died.

For those patients who had an ELISA response greater than 100 picograms per mL as a continuous variable, we found that there was a P value for association or correlation of relapse free

interval, or relapse resurvival, with a P value of .003 based on a Cox proportionate hazard model.

And, again, I don't mean to overplay this. It's a very small trial. We only had 22 patients with immune response data, but the bottom line is if you had a very good ELISA response, those are the patients who are alive NED. All of the relapsers had either no or a lesser response, and that's just a hint that there may be some value to this immune response assay.

Conclusions. Immune responses by ELISA in ten of 22 patients. We saw 12 of 22 who had positive DTH for the MART peptide, but only three of 11 correlated with the ELISA.

Meaning of the DTH to me was unclear. Again, nine of 25 relapse with three deaths. The toxicity was minimal. The correlation, there was certainly a hint of some beneficial effect, but that remains to be seen in follow-up trials.

Let me quickly switch gears, and then we'll go on to an important question based on some data we've heard before, and again, a strong rationale to be asking questions about peptide pulsed dendritic cells as an immunogen was that data from a variety of labs, including Mike Lotze whom we

heard yesterday, suggested that dendritic cells were potent antigen presenting cells.

There is also data from a number of labs suggesting that you could grow dendritic cells in large numbers derived from peripheral blood mononuclear cells by the expedience of using IL-4 and GM-CSF, setting up a hypothesis that potentially tumor antigen peptide pulsed dendritic cells could induce potent antitumor immune responses and hopefully a clinically beneficial effect.

So our goal in a trial that started about a year ago was to treat up to 20 patients with Stage IV melanoma with measurable disease with up to 100 million dendritic cells derived from peripheral blood mononuclear cells, pulsed with multiple peptides from melanoma antigens.

We set out in the classic Phase I style to evaluate the toxicity, whether there were immune responses, and since we chose patients deliberately who had some measurable disease, we would be able to look at clinical responses.

And the overall goal was to refine techniques for the generation of large numbers of potent immune stimulating dendritic cells.

Demographics. So far 11 patients treated. We just accrued the 12th, and that's about a patient a month over the last year. Four of them have actually had four cycles of dendritic cells. Typical patients for our population, five women and six men.

Everyone had visceral disease. They were all HMB-45 positive because that's the antigen that is actually -- that's the antibody that actually recognizes the GP-100 antigen.

Several of them actually had never had systemic treatment, and two of them actually had ocular or choroidal melanoma primaries.

The schema, very straightforward. As Steve discussed, we used the GP-100 210M substituted peptide and the tyrosinase 370D substituted peptide, which are found to be immunogenic.

This was a Phase I trial with two injections of dendritic cells given intravenously two weeks apart, starting at ten to the seventh cells, moving on to ten to the eighth or, if possible, if practical, three times ten to the eighth.

The endpoints initially were toxicity and immune response. We did a leukophoresis with

skin testing just prior to the first infusion, and then four weeks after the second infusion, and patients, if they responded, we asked if they could be retreated.

And again, they were not toxic as one might expect. No irreversible Grade III and no Grade IV toxicity. No change in any hematologic or chemical parameter related to therapy.

One of the patients who got three times ten to the seventh cells had some pretty impressive myalgias and arthralgias for several days after both of the infusions.

One patient had high fevers and fatigue. We called that transient Grade III toxicity, but overall the investigator and the patients agreed that these peptide pulsed dendritic cells were well tolerated in general.

The schema. We ficolled the leukophoresed PBMC. They were adhered to plastic as implied by Ralph Steinman and Jacques Banchereau.

We then removed the nonadherent cells, and the adherent cells were grown for eight days in AIM-V serumless media with 1,000 units of IL-4 and GM-CSF. Twenty-four hours prior to harvest, we added peptides in separate aliquots to the cells,

and on day nine we harvested the cells, and actually we irradiated the dendritic cells prior to their administration.

They were put in a transfer bag with some albumen to stabilize them and intravenously infused over 15 minutes.

We did gram and fungal stains. We did the usual QA/QC things. For example, bacterial and fungal cultures were sent as they were infused. We did endotoxin mycoplasma assays in cultures, and we locally developed all of our SOPs and performed this in a dedicated room at our cancer center.

And, again, as other people have shown -- I won't harp on it -- in forward and side scatter these are very large cells in general that are HLA-DR positive. If you gate on the large cells, they're predominantly CD-86 positive in our hands; again, DR positive; CD-54 and CD-58 positive.

And, again, our cells are somewhere between an immature and a mature dendritic cell because they are relatively CD-83 positive.

And again, yields in phenotypes. The bottom line is on the bottom line, and I would just look at the yellow bottom line. Our cells turned

out to be across the board about 50 percent dendritic cells.

And again, one can argue over what defines a dendritic cell, but it's a cell that we called CD-14 negative, 58 positive, 86 positive, DR positive, and if you average these together, you came up with about a 49 percent purity.

The viability of our cells, and this just describes how many we infused, when we made them fresh we then froze an aliquot and infused the frozen and thawed aliquot two weeks later. The viabilities here were 85 to 90 percent. Here the viabilities were somewhat less. They were, of course, frozen and thawed cells.

And the bottom line in terms of the immune responses, this is an ongoing trial and so far we've looked at five patients. Peter Lee, who will talk later, is looking at the tetramer assays.

We're doing the same kind of restimulated PBMC cytokine release assay that was done for the MART trial. So far only one of five patients has had any evidence of immune reactivity.

Again, cytokine release against flu, meaning that the patient is flu competent, against GP-100 suggesting that there is evidence of boosting

against GP-100 pre as opposed to post, and somewhat weaker boosted immunity against tyrosinase, again, pre as opposed to post, and these are all T-2 cells pulsed with the appropriate peptides, either none, flu, GP-100 or tyrosinase.

This turned out to be a patient who has had evidence of tumor aggression, by the way, and continues to be treated, and this just shows you the usual anecdotal patient. The fifth patient or sixth patient in the trial actually had multiple pulmonary nodules and was a partial responder after cycle one of the dendritic cells. That just shows a pulmonary nodule on the left side going away, gone.

She also had a very small nodule down here, difficult to make out, there, there, gone. That, by the way, for the nonclinicians is the top of the right diaphragm.

The patient also had another pleural plaque that was about four centimeters that disappeared after the second infusion, and this is a patient who actually seems to be a complete responder after the second cycle of dendritic cells.

And, of course, in answer to the usual question, it's a patient whose cells we have collected both pre, post cycle one, post cycle two.

I honestly will not know the answer to the immune assays for another week or two.

This is another patient, actually the first patient we treated, who had very indistinct looking liver lesions that were biopsied positive for melanoma. He had multiple lesions. Most of them shrank down significantly. He was about a 48 percent regressor. So he just missed being a PR. He was scored as an MR or a minimal response.

Clinical results. Eleven patients treated at varying and increasing doses for two injections each. Two patients had minimal tumor regression, not quite meeting the criteria for PR.

One CR with multiple lung mets. Eleven are alive, ten with disease. We've done DTH testing. No one has responded to GP-100 and tyrosinase. Everyone has had a positive DTH control to candid. or mumps.

We have seen evidence of augmented immunity by ELISA only in one patient. this one patient just happened to be a patient with multiple lung mets. who has had a minimal response.

We'll get more data about the patient who had the complete response in the next couple of weeks.

Our plans for the future involve using CD-40 ligands to now activate the dendritic cells and hopefully make them better antigen presenting cells, which can be shown in vitro.

We'll be working with Jim Mule also to utilize low doses of IL-2 as an adjuvant post dendritic cell infusion based on some of the nice work that he's done and has already presented this morning.

Now, in the last couple of minutes let me very quickly switch gears and finish up. We've also performed a peptide trial in patients who have HPV induced preneoplasia, and again, as someone mentioned previously, this is an excellent tumor specific antigen as opposed to a neoantigen, which is most of what the melanoma antigens are.

HPV-16 and 18 are implicated in the majority of cases of high grade cervical and vulvar interepithelial neoplasia, which is a clear precursor to cervical cancer. As we've already heard, they encode E-6 and E-7 transforming proteins which contain immunodominant peptides restricted to HLA-2.1.

There are a number of peptides that Martin Cast, Cass Malief and others have shown can

be used to generate specific CTL ex vivo. The rationale for our trial is that most cases are 16, 18, 31, 33, 45 positive.

Since the high grade HPVs were implicated in causing high grade CIN/VIN, then hopefully if we could vaccinate patients using peptides from HPV E-7 we might resolve or prevent viral infection, and that would be a strategy potentially to prevent cervical cancer.

And the idea was to immunize against HPV-16 E-7 to generate a T cell response and ask whether we could eliminate the virus.

And, again, very simple schema. We chose women with high grade CIN or VIN, which is vulvar interepithelial neoplasia. They had to be HPV-16 positive by a sensitive PCR assay, and of course, HLA-2 positive.

We gave four doses of an HPV E-7 12 to 20 peptide vaccine with incomplete Freund's adjuvant prior to their definitive therapy for their CIN/VIN. So we delayed their definitive therapy by four months, and we gave doses at 100, 300, 1,000, and 2,000 micrograms every three weeks times four.

We actually had, courtesy of the NIH, the E-7 86 to 93 lipopeptide available, and that

began to be added after patient number 11, and, again, now we have a clinical endpoint or at least a clinical surrogate. We have disappearance of virus as an endpoint. We have again the familiar immune CTL response assay, and now we have another clinical endpoint, regression of dysplastic lesions by biopsy.

And we've seen, again, minimal toxicity, one patient with local pain. It's really been again, according to investigator and patients, very well tolerated vaccine therapy.

And, again, these are the immunologic assays. We've actually treated more patients. It's really more of the same. So I only show you the kinds of cytokine release assays that I showed you before. They're grouped in pre/post pairs, pre/post, pre/post.

If you look at this column here, it just shows the flu. Virtually all of the patients were flu competent.

If you look at this column, and I won't belabor the point, it shows that of the first ten, six of them had evidence of boosted immunity. Typical patient here, here, et cetera.

We've done 12 patients' immune assays. Seven of the first 12 had evidence of boosted immunity, but to end up what we found is that when you look at viral clearance, there was not a clear correlation between the immune response assays and the clearing of virus.

There is also not a clear correlation between the immune response assays and pathologic clearing of the lesions which occurred in three of the patients.

To our surprise, we sent PBMC to Martin Cast and found again, surprisingly, that TCR zeta chain expression was severely reduced in the PBMC of these patients, and again, a very surprising finding.

Results and conclusions from this trial to end up. So far we've treated 15 patients. We just added 16 and 17 this past week. Three our of 12 have complete disappearance of their lesion. Seven out of 12 had increased E-7 specific immunity by chromium, and we've confirmed this or by cytokine and we've confirmed this with chromium release assays.

Seven of the 12 had disappearance of the virus by PCR assays for up to six months. There was

no clear-cut correlation between the disappearance of virus, the disappearance of the lesion and these immune response assays. It's not been toxic, and no one has progressed to invasive disease, which is an important thing to measure.

Conclusions overall based on snapshots from three clinical trials. Yes, you can measure boosted antigen specific immunity by doing cytokine release assays. These are very difficult assays to do. They're labor intensive. They're hard to reproducibly quantitate.

There's a smidgen of evidence from the initial MART trial that there's a correlation between the post vaccine gamma interferon release as a continuous variable and the favorable clinical effect of relapse resurvival.

This is not an assay that's ready for prime time. I think the overall over arching point is that we need to come up with a better immune assay where there's a clear correlation to a beneficial clinical effect. I do not think we should develop immune response assays in a vacuum.

Finally, let me conclude by thanking my collaborators: my group of technicians, my data managers and research nurses, my GYN oncology

colleagues, Mario Sznol from CTEP, Jay Greenblatt, Jan Morgan who made the peptides available, and Mary Ellen Rybak, who's I believe here from Schering who kindly made available IL-4 and GM-CSF for the dendritic cells.

Thank you.

(Applause.)

DR. SIEGEL: Okay. We're now breaking for lunch. I'd like to stick with the time for reconvening at 12:30. So I'd ask you to try to eat expeditiously. We'll start up again on schedule at 12:30.

(Whereupon, at 11:46 a.m., the workshop was recessed for lunch, to reconvene at 12:30 p.m., the same day.)

AFTERNOON SESSION

(12:33 p.m.)

DR. LEE: Good afternoon. I'm Peter Lee, and I'm a Fellow at Mike Davis' lab at Stanford, and it's a real honor for me to be speaking today.

In the next few minutes, I'd like to tell you about a new way of studying antigen specific T cells that could be particularly useful for monitoring the immune response to cancer vaccines.

In this meeting we've heard a lot of exciting data about different vaccination approaches. However, so far no strategy has given a 100 percent response rate, and why some patients respond while others don't to the same immune intervention remains largely a black box.

The more that you can understand what's going on inside this black box, the more quickly you can devise better vaccination strategies. The important questions include not only what is the magnitude of the response, the quantity, but also the quality of the response. What are the functional characteristics of the cells? Are the cytolytic in vivo?

What are the phenotypic characteristics? What surface markers do they express? What cytokines do they secrete? And what is the temporal dynamics of the response? Do the cells come up quickly or disappear quickly or do they come up gradually but stay high?

These are parameters that are important for cancer chemotherapy. So it would make sense that they would be important also for cancer immunotherapy.

The current methods of getting antigenic T cells mainly include the LDA, limiting dilution analysis, and ELISPOT. Now, these methods mainly address the quantity of the response and not really the quality. They're both sensitive and specific, but they're also labor intensive and time consuming, making it difficult to screen a lot of patient samples at multiple time points.

In addition, the LDA detects only those cells that remain proliferative and cytolytic in vitro and, therefore, could miss a lot of cells that for whatever reason don't proliferate in vitro, and in fact, a number of recent studies have shown that the LDA underestimates the true number of antigen specific cells by anywhere between ten to 50-fold.

Likewise, ELISPOT detects only those cells that secrete the cytokines that you're looking for and, therefore, could miss cells that either secrete cytokines that you didn't expect or just don't secrete cytokines at all.

And so if you're using solely these two methods to monitor your immune response to your vaccine, you may be getting a gross under estimate of the true picture.

A very important point is that both of these methods don't allow further analysis of antigen specific T cells. You can't use them to isolate these cells out and study them further to understand the biological activity properties, and because both methods require significant in vitro stimulation which could otherwise activate or alter these cells in vitro, they don't tell you what's the native in vivo state of these cells in the patients, which could be a very important question to ask when you're monitoring your clinical trial.

So what can you do to make it better? Well, we know that the T cell receptor binds to MHC peptide molecules. So is it possible to make soluble MHC peptide molecules to stain antigen specific T cells through the T cell receptor?

It turns out that this interaction has a fairly low affinity, approximately 1,000-fold lower than antibody antigen interaction. So if you were to make soluble MHC peptide molecules, they would simply fall off during the wash and you would get no staining.

John Altman, a previous postdoc. in our lab who's now at Emory, found a creative solution. He engineering a biotinylation signal peptide at the end of the MHC molecule which allows you to biotinylate the MHC and by adding avidin, you can then bring forward these monomers together into tetrameric complexes.

These complexes can engage two or three T cell receptors simultaneously, thus greatly increasing the avidity of the interaction and making staining possible.

Very briefly, the way that these reagents are made is that the beta-2-microglobulin and the MHC molecules are synthesized in E. coli. The peptide of interest is synthesized by a machine, and these are mixed together in a folding reaction which goes over three to four days.

At the end of this, a very small fraction will be properly folded in a trimaric

complex. They are biotinylated with the enzyme VRA and then extensively purified using FPLC and mono-Q.

At this point you add avidin at the correct molar ration to bring these together into tetrameric complexes, and by using avidin that's directly conjugated to different fluorophores (phonetic), such as PE or APC, you can use these as staining reagents for FACS analysis or sorting.

There are certain limitations to this approach. First of all, it requires you to know the exact peptide target. However, in cancer vaccines, that may not be such a big limitation because oftentimes your vaccine is your peptide target.

Tetramers do require a fair bit of experience to make, and therefore, they're not widely available yet, and the sensitivity of this method is limited by the sensitivity of FACS analysis, and we've gotten our limit of detection down to approximately .02 to .01 percent, or approximately one in 10,000 cells.

So now let me show you a few brief examples of what you can do with the tetramers, looking at patients with melanoma and cervical cancer.

In collaboration with Jeff Weber at USC, we've looked at some patients with cervical cancer who were vaccinated with the human papilloma virus E-7, 12 to 20 peptide. Here are examples of two patients that responded immunologically.

On the X axis is CD-8 staining that were gating around CD-8 positive cells. On the Y axis is staining with a tetramer, which is made of HLA-A2.1 in association with the E-7, 12-20 peptide.

Prevaccine, both patients had essentially no tetramer staining cells. Our background is .01 percent or less.

Thirty days post vaccine, Patient A had a significant increase in the tetramer staining cells to .21 percent of CD-8 or approximately one in 500.

Patient B had a much more subtle response, representing only about .03 percent of CD-8s. However, you can see that this population is quite distinct and discrete, making this data fairly believable.

I should say that in talking to Jeff that neither of these patients had any clinical response to the vaccine, suggesting that the mere appearance of peptide specific T cells may or may

not be sufficient, and so we're in the process of sorting these cells out and further analyzing their biological characteristics to understand why they're not doing the job.

Not only have we found evidence for potentially tumor reactive T cells in vaccinated patients, but we've also found them in some non-vaccinated patients. This is an example of a patient with metastatic melanoma prior to any therapy or vaccine.

We've stained this patient's PVMC with a panel of three different melanoma tetramers made with MART-27, GP-100-154, and Tyrosinase-368, and we found a very prominent tyrosinase specific population in this patient, representing over two percent of all the CD-8 cells.

Remind you again that this patient is completely unmanipulated, no vaccines.

This patient also did not have any evidence of vitiligo to suggest that this is somehow a coincidental autoimmune process.

In addition, this patient had a small EBV, Epstein Bar virus, specific population representing about .2 percent of the CD-8 cells.

One important thing that we can do with a tetramer is combine it with a lot of different antibodies to know what are the other markers that are being expressed by these antigen specific T cells. We've stained this patient's cells with a panel of anti-human V-beta antibodies, and we've found that of all the patient cells that stain with the tyrosinase tetramer, they all stain with a single V-beta antibody V-beta-20, strongly suggesting that this population is monoclonal at least with regard to V-beta.

In collaboration with Mario Rhoederer at Stanford, we've coupled the tetramer methodology with a nine color FACS system, which really allows us to look at a whole host of different markers that are being expressed by these cells to get a very complete picture of the phenotypic characteristics of the cells.

And so far we've looked at over 30 different surface and intracellular markers that are being expressed, including markers like CD-45 RA and RO, which help delineate the T cell subsets, activation markers, such as CD-38, and other markers, such as the NK marker CD-16.

One of the cool things we can do is directly compare two different antigen specific populations from the same patient simultaneously, and showing here at the tyrosinase specific population and the EBV specific population, and it turns out that they're very different phenotypically.

Whereas the tyrosinase specific population expresses CD-45 RA but not RO, the EBV population was the reverse. They express RO and not RA, which is a more classic pattern for memory T cells.

The tyrosinase specific population expresses low levels of the activation marker CD-38, while the EBV population does not, suggesting that this population may be partially activated in vivo.

And interestingly, this population expresses low levels of the NK marker CD-16, which suggests that these cells may have some NK-like properties or that they're NK-like T cells.

Another very important thing that we could do is isolate by sorting these two populations independently and directly assay them for the cytolytic activity without any in vitro

manipulations. The EBV population had strong cytolytic activity against peptide pulsed targets.

The tyrosinase specific population was completely noncytolytic against either peptide pulsed targets or melanoma targets, strongly suggesting that this population is noncytolytic in vivo, and this may be the explanation why the patient's melanoma progressed despite the existence of this large, potentially tumor reactive T cell population.

And this, I think, is a particularly important point for this audience because so far probably the main goal for cancer vaccination has been to elicit a tumor reactive CTL response, but this data strongly suggests that having this population may or may not be enough; that you also have to make sure that this population maintains its cytolytic activity, kind of like what Dr. Banchereau said yesterday. It would be like these crocodiles turning back into zebras in vivo.

And potentially there may be a number of theoretical reasons why tumor cells may be able to change the phenotype of tumor reactive T cells in vivo, and I think that could be a very important point for future vaccination approaches.

Because this method is relatively easy to do, we're able to study multiple time point in this patient to get a sense of the temporal dynamics of the response.

This patient was treated with a total of four cycle of chemotherapy as indicated by the arrows for metastatic disease. This population dropped precipitously after the first cycle of chemotherapy and remained very low throughout, even well after the chemotherapy was discontinued.

What's not shown on this slide is that the EBV population did not change at all with chemotherapy, suggesting that these two different antigen specific T cell populations have different sensitivity to chemotherapy in vivo.

This could reflect the fact that the potentially tumor reactive T cell population is more active in vivo and, therefore, more susceptible to the effects of chemotherapy, or that somehow they're primed for the apoptosis pathway.

So in summary, the tetramers offer a number of advantages that could be very useful for monitoring immune responses to vaccines. First of all, it doesn't require the cells to remain functional in vitro and, therefore, gives you a much

more accurate enumeration of the total number of antigen specific T cells, and you can also get a sense for what percentage of those cells are functional or not functional.

It allows you to directly isolate these cells by sorting so that you can further analyze them, and also you can expand them in vitro for adoptive immunotherapy.

You can couple the tetramers with a whole host of different antibodies, both surface and intracellular markers, with multi-color FACS analysis to get a much more complete picture of the phenotypic characteristics of the cells.

And finally, because it's relatively easy to perform, it allows you to quickly screen a large number of patient samples at multiple time points to get a sense for the temporal dynamics of the response.

So going back to the black box that I originally posed for the beginning, you know, you have your vaccination strategy, and you have clinical response. The tetramer is really going to be a very useful method to start dissecting out what's inside this black box, to understand what are the immune characteristics that lead to good

clinical outcomes, and then to screen different immune strategies that could elicit those desirable immune responses.

I just want to acknowledge Mark Davis, my PI and mentor at Stanford; Mario Rhoederer, who's a real FACS whiz at Stanford, who helped develop the nine color FACS system; Cassian Yee and Phil Greenberg at Seattle, who are very close collaborators for us; and Jeff Weber at USC, who's collaborating with us to study the different vaccination approaches that he told you about earlier.

Thank you.

(Applause.)

DR. SZNOL: Thank you, Dr. Lee. That was a very elegant talk.

I'd like to move on with the agenda, and I'd like to introduce Dr. Kim Lyerly of the Duke University Medical Center to speak about assays for monitoring a CEA peptide induced immunologic response in a dendritic cell trial.

DR. LYERLY: Thanks, Mario.

I want to again thank Raj and the organizers for really putting together, I think, a wonderful program, and it's a real privilege for me

to be here to chat a little bit about the program and our particular interest in DC based vaccines.

Could I have the first slide, please?

What I'll start with is really to acknowledge the fact that although I wish I could be a dendritic cell evangelist, I guess I'm more of an agnostic right now, and what I'd like to do is really see what the experiments will show us and really determine what kind of data we can get that we can elicit T cell responses of a magnitude and durability that we think they may have some clinical benefit.

Now, this slide is a cartoon that just depicts in very simplistic terms what we all think may be some holy grail, some cellular immune function.

Again, not to belittle the contribution of antibodies, let's say that there's some perhaps functional activity that's good for us, and what we have to achieve is a super threshold level of this functional activity in which, in fact, we'll have a clinically effective response, and I'm focusing on T cell responses during my talk.

What we can see here is that rather than the simple enumeration of a digital response, a

responder versus a nonresponder, we actually have to perhaps look at the quantitative response and the durability of these responses because if they are clinically ineffective, they may, in fact, lead us to choose something that if further developed could be more effective.

And what I haven't included in this slide, which is, again, very, very simplistic, is the fact that the magnitude and duration of the response during active disease or during a period of effector cell function may be quite different than the type and magnitude of the response during the memory cell function.

So, again, I think I just want to highlight that we're really poised to answer a lot of questions with some new technologies, but we're trying to be very receptive and say we're going to be as unbiased as we can in trying to measure some of these things.

So what I'm going to do is focus, based on reading the program and kind of thematically being consistent with this session, is to look at some of the post immunization analysis issues that we think are important.

The tetramer talk that we just heard, I think, was a beautiful description of that technology, and I think the place that it really is the state of the art, and I would say that it really fits into the category of direct analysis of cellular responses, again, in this case the circulation, where, you know, the phenotype or the TCR that's specific for that specific peptide can be quantitated by flow.

And there's another type of analysis that is quite interesting, the immunoscope out of the Pasteur Institute, and I'll show you some data on that.

The other forms of direct analysis that I'll spend some time talking about are more perhaps functional assays, but they require a stimulation phase, and again as pointed out, they do alter the cell that we're trying to measure, and this is the Fastimmune assay that we use, which is a three color flow based assay that looks at intracellular cytokine expression.

Now, as you can see, I've actually segregated the assays from direct to in vitro stimulated assays, and I think the in vitro stimulated assays are probably the state of the art

today. They require activation, say, usually antigen or APCs, and even though dendritic cells may not be added to the in vitro culture, the fact that there is a low frequency of APCs within the PBMCs is probably the APC source here.

Oftentimes we take days, perhaps to weeks, of in vitro stimulation, and often the unspoken is that there are accessory growth factors added to these cultures, and they may be pharmacological additions of defined cytokines like IL-2 or IL-7 or IL-12, or they may actually represent the fact that CD-4 cells are present. They're being stimulated and are producing cytokines that are sustaining growth of the antigen specific T cells.

And then the assays are typically performed, and again, something that's probably unspoken is the fact that timing of these assays is critical. If you actually restimulate antigen specific T cells too quickly after an in vitro priming, you'll probably reduce the type of response you get. You may trigger a ptosis rather than activation or cytokine release and so forth.

So as you might imagine, there's a huge variety of parameters just in the in vitro assays

that require in vitro stimulation, and we are focusing a lot of energy in trying to avoid all of those by direct analysis.

One of the things that we've tried to also do is try to come to some sense of what's the magnitude of the responses that we're likely to see versus the types of responses that we all can form some consensus on that are therapeutic, and some of those therapeutic responses may, in fact, be T cell responses against well known pathogens in which T cell immunity is known to play a role.

So as an example, we looked at EBV specific T cell responses, and if we do ELISPOT analysis of the peripheral blood from healthy donors that are EBV seropositive, again this cartoon depicts this type of analysis, an ELISPOT that depicts a circulating frequency of EBV specific T cells.

And as an example, we can actually quantitate this using in this case the EBV transformed autologous B cells as a target cell and as an APC. They serve that unique role, and you can see a very nice segregation of those responders.

We can also use this type of analysis, called the Fastimmune, which is three color flow in

which we stain in this case CD-4 positive cells that are CD-69 positive, and we can gauge on TNF alpha or interferon gamma secreting cells, in this example, and identify populations of cells that are responding by the intracellular accumulation of TNF alpha or interferon gamma in a fairly reliable and reasonable fashion.

We can also use this form of analysis to look at CD-8 positive cells, and this is a population of cells that were stimulated in vitro and shown to be cytolytic, and again, analyze where we see again a population of CD-8s that are cytokine secreters.

Now, again, this seems very simple. You use three color flow, and you use brofeldinate (phonetic) to prevent secretion and just use antibody staining for intracellular cytokines, but again, the unspoken is that there's a variety of physiological changes in the cytokine as it resides within the intracellular compartment, and the pH changes change the confirmation. So you have to screen a large variety of antibodies.

So the antibodies that bind to soluble cytokines in an ELISA may not bind very well in

these Fastimmune assays. You really have to look at all of these details.

So let's switch to another system that doesn't have an APC and a target cell within the same cell. Here's a CNV specific response, and if we take CNV seropositive donors and generate CTLs in vitro and we subject the V-CTL populations to the same type of analysis, you can see the traditional cytolytic assays where we have a nice EDT titration of killing.

We can analyze those same populations to see frequencies of CNV specific T cells that are secreting in this case gamma interferon, and you can see that if we do the Fastimmune assays, gating CD-4s or CD-8s, we get populations of T cells.

What's very provocative is that although we can get nice killing here, the actual frequency of CD-8 positive cells secreting cytokine is quite small compared to the CD-4s, and in fact, again, can give us some insight as to the nature of the immune response that appears to be much more informative and quantitative than the typical cytolytic assays that we tend to use.

This is an example in which not only can we use a gamma interferon, shown in the left-hand

panel, but can actually use intracellular cytokine antibodies to IL-2, TNF alpha, and IL-4, again giving us the opportunity to look not only for TH-1 type of responses, but in this case TH-2 type of responses.

And I want to point out that these assays, as nice as they look, they do require a lot of work. Yu Ping Dang and Paul Mosca in the laboratory worked very hard to actually develop an assay that we can use on cryopreserved blood in a direct, six hour assay using APCs and some physical separation methods to detect in this case an antigen specific CNV T cell response gaited on CD-8 secreting TNF-alpha and interferon gamma.

So I use these types of examples, again, to illustrate that probably in our hands the functional state of the art in which we can detect antigen specific T cells, that we have a fairly good level of confidence in that they do serve some clinically beneficial role to the host.

So let's move on to how can we apply these same types of principles to analyzing T cell responses in our clinical trials. This is actually the house that Eli Gilboa built at Duke, and I want to acknowledge, you know, his contribution as a

collaborator and a friend in helping us move into the field of dendritic cells, and in fact, this building was built under his guidance to house a GNP-GLP facility for the processing of dendritic cells based on much of the work that he's done in generating T cell responses in animal models against peptide and RNA modified dendritic cells.

So, again, based on some of the feedback I've taken from yesterday's sessions, let me just point out that we started clinical trials with peptide pulsed and RNA pulsed dendritic cells using a single defined antigen called CEA. Jeff Schlom will probably talk about that a little bit later.

We used the monocyte derived DCs from cancer patients after phoresis growth in serum free media in GM-CSF and IL-4 kindly provided by Mary Ellen Rybak from Schering.

Then we actually washed, pulsed with antigen, and actually we cryopreserve the entire lot of dendritic cells that we generate because we wanted to do some characterization of these cells before we administered them.

We looked at the typical sterility and viability issues. We stained for these markers only because we wanted to use a panel of other markers,

but these markers we actually could get performed in our bone marrow transplant lab under GLP conditions, and we actually looked at function, not as lot release criteria, but as a research assay for the cells that we were giving back.

This is a very busy slide, but typically it's going to give you two groups of cells, one in which we just generated dendritic cells based on that protocol, and you can see we get the typical yields, and these are all in cancer patients.

We also used -- actually that's flipped around -- we also used a strategy in which we did an antibody depletion at the final step to remove that contaminating population of small cells found in all the dendritic cell preps. to try to improve our yield and the purity of the product.

This is, again, the depleted and the nondepleted cells, the typical histograms from the flow cytometry. You know, we see a little bit of contaminate 8-14. We see 86 and DR at fairly significant levels, and these are the typical yields.

And what's interesting is although we've spent, you know, about $2,000 worth of columns and antibodies to deplete, we didn't really get, you

know, an amazingly better product. So some of the features of immunophenotyping for dendritic cells that allow us to give more pure or an apparently more pure population, I think, will rely a little bit on defining the parameters, defining the features of dendritic cells that we want.

And I agree completely with the discussion from yesterday which is it's hard to define that ideal population, and we have no idea that in this depleted population if we completely removed the cells that were truly affecters or truly beneficial. So we've actually stopped doing this antibody depletion step.

The other point I want to make is the issue of contamination of the phenotype and the assessment of the maturity of dendritic cells. This is just, you know, two dendritic cell preps. in serum and serum free conditions in which we looked at CD-83 expression based on exposure to TNF-alpha.

And you can see the hours after exposure. You see a shift in the CD-83 expression, again, consistent with the ideas that CD-83 is intracellular and being presented on the cell surface as the dendritic cells mature, and this change in phenotype also changes in association with

the functional ability to take up antigen and present.

So, again, I worry a little bit if we're presuming that we know what type of cell to give we'll set some arbitrary conditions for what's real, and in fact, I would suggest that if, you know, you say that you have to have 90 percent CD-83 positive cells, you may bias all of the lot release criteria to the people with the dirtiest labs because they'll have a lot of contamination. They'll have some cytokine release from granulocytes or some contaminating cells, and in fact, that's probably not what you want to do.

Well, we also did the functional assays on all of these patients. We actually did primary T cell responses. This is work done by Smeda Neyer and Mike Morris in the laboratory in which they actually spent two to three weeks of in vitro culture demonstrating that in all of these cryopreserved dendritic cell preps. in these A-2 positive patients, we were able to generate T cell responses specific for the peptide, some greater than others, and you can see there's a variety of levels of cytolytic activity.

Again, I show this slide not to suggest that we should be doing this, but to really emphasize how important it is to search for alternatives rather than the traditional cytokine or cytolytic function, again, which is very labor dependent and depends on high levels of IL-2, IL-7, depletion of CD-4 cells, and then an in vitro assay for lytic activity against a target cell.

What this really means is unclear. The same type of analysis on the RNA transfected cells.

Again, what I want to do is highlight the contribution of Eli Gilboa in his observation that RNA transfection of dendritic cells can elicit primary T cell responses, and this is a CEA specific cytolytic activity correlating with this Fastimmune type of analysis, which again perhaps may be a functional assay that can replace some of the cytolytic assays that are done.

So for the last two minutes of the talk, I'd like to spend some time talking about the actual clinical trial, which is using these cryopreserved dendritic cells. We gave them IV. We actually did a couple of studies that are present in cancer research after indium labeling showing that IV

administration leads to a distribution to the lungs and then to the spleen and the liver.

And what I don't have for the lack of time is the fact that if we do subcutaneous versus intradermal injections, the radiolabeled dendritic cells in the injection to the dermis appear to traffic to the draining lymph nodes, but they don't appear to traffic when they're injected subcutaneously.

Now, again, we have no idea if the draining lymph nodes are attracting the dendritic cells that are truly triggering T cell responses, and again, those will form the foundation for further studies.

Okay. Let me point out that we have been very adamant about getting prevaccine phoreses samples as baselines, undergoing vaccination, and appreciate that circulating and trafficking T cells may, in fact, be different cell populations.

Here's an example where if we take a post vaccination sample and do a CTL expansion we get some activity. It appears to be a little bit higher than the prevaccination activity, but what's even perhaps a light bit higher is the T cells associated with tumor that are isolated from

malignant infusion, again, suggesting that if we look in the peripheral blood only, the frequency and function of the T cells, that in fact may be useful in providing some clinical benefit that, you know, may be quite different.

Again, to highlight the fact that one of the advantages we think of whole protein or complete tumor antigen loading of dendritic cells is the fact that CD-4 responses can be obtained, and this is with an RNA transfected DCs stimulating T cells.

And let me spend, again, some time talking about the T cell analysis. Here is a peptide pulsed dendritic cell patient, again, ELISPOT analysis, very low frequencies of T cells, but maybe some hint that there's some T cell activation in the post immunization sample.

If we do a TCR analysis comparing pre and post immunization, there's some hint that V-beta-14 and V-beta-21 populations of T cells may be expanding, and these are direct analysis of RNA isolated from peripheral blood.

And if we actually do in vitro stimulation of normal donors, as well as looking at some of our patients, we see in some of those who have had other clinical or other immunological

parameters of T cell responses in the peptide some sense that there may be some oligoclonal expansion.

What's interesting, this data is very different. This family, the V-beta-21 family, is very different than what has been reported for restriction by Rumella Philip. You know, I can't explain that, and this is the confirmatory cytolytic activity, again, after two to three weeks of in vitro stimulation, showing killing of CA specific targets.

In my last 20 seconds, I want to point out that obviously the holy grail is data such as this in which we can identify immunological surrogates that will predict positive clinical benefits, and I would say that in my opinion we'll have to look for prolonged life or an absence of recurrence.

This is, again, an illustrative example of other surrogates, you know, trying to correlate with the surrogates. Obviously we can see the folly in trying to do that, but at some point in the future we will hopefully be able to develop these surrogate markers, the activation of specific T cells in a measurable fashion that will hopefully correlate to the development of positive or

beneficial clinical responses, in this case CA-15-3 that drops back down into a preimmunization or preregression level.

Again, the one thing I wanted to highlight, Eli Gilboa had a poster here yesterday. The one thing we like about the RNA in the group is that we agree a lot with Jim Mule and some of the other whole tumor cell vaccine people in that we're not exactly sure which tumor antigen is the authentic tumor rejection antigen, and the potential use of RNA may overcome some of the problems with isolating whole tumor cells from every patient.

Jeff Sosman was saying, "Well, you guys, autologous tumor cell vaccines will never work because you'll never get enough autologous tumor cells from any, you know, great population of patients to make this work. The only thing you'll be able to do is get a paraffin fixed slide, and that's the only source of tumor antigen that you're going to have."

And, in fact, the use of RNA actually overcomes that obstacle. In fact, you can use the RNA content from cells within the paraffin fixed slide to generate messenger RNA encoding for all of the antigens within the tumor, and as a proof of

principle, Eli, Smeda Meyer, and the clinical group isolated the tumor from patients along with the corresponding phoresis.

You can see here that the dendritic cell stimulated with CEA and GFP as a control were able to target RNA transfected DCs, autologous tumor cells, and DCs transfected with total tumor RNA.

And, again, in my last slide, it's very interesting. If you take the total tumor RNA content of that tumor cell, and this is from Eli's poster, you actually can generate a T cell response in vitro that's cytolytic for autologous tumor cells.

So, again, I say we're at the beginning of an exciting period of time. I want to, again, thank the organizers for giving me the privilege of showing you some of our data.

Thanks.

(Applause.)

DR. SZNOL: Thanks, Kim.

The next talk is by Dr. Mary Disis from the University of Washington on peptide-based vaccines for cancer immunotherapy.

DR. DISIS: Thank you.

Basically my mandate was to talk about laboratory monitoring, and I'm going to follow up what Kim was talking about, not necessarily showing you the panoply of very quantitative assays that are coming out right now to monitor clinical trials, but rather show you a snapshot of an assay that we use in the lab and the struggles with trying to determine sensitivity and specificity and how assays correlate to a gold standard in a field where there really is no gold standard.

So firstly, what I'd like to do is talk a little bit about clinical trial design in terms of the patients that we're immunizing, and really the big problem of trying to immunize patients with cancer with a vaccine and exactly what's going on with their immune systems and are they really immune competent, and just a little bit of data that we're trying to collect on the patients that we're immunizing, and really spend the bulk of my time taking you through a particular assay and showing you how we're trying to compare it with other assays in the lab.

And I'll start by telling you my bias that I really think we're at a point in time when there are a lot of tools available to us, and

inherent into Phase I studies should be people who are willing to compare assays in a fashion uniformly in multiple patients over time so we can develop the type of database to tell us what the sensitivity and specificity of these assays are.

I'll also start by telling you that I'm going to talk about an assay for looking at CD-4 T cells, and in fact, our current Phase I trial of HER-2/neu peptide vaccines of, which I'm not going to talk about clinical data and the results we're seeing, is to really immunize patients with vaccines that are composed of three peptides.

And these peptides are longer helper epitopes that have been figured out in experiments that have already been published to be epitopes that will elicit a helper response to HER-2.

The structure of the HER-2/neu antigen -- it's a transmembrane domain protein that consists of an extracellular domain and intracellular domain -- really lends you to think that not only a cytotoxic T cell response may be effective, but also an antibody response, and indeed, people have already shown that antibodies directed toward HER-2/neu can be clinically effective. So this is our strategy of immunization

with longer 15 MER peptides that are potential helper epitopes.

We do have an immunization strategy going for eliciting HER-2/neu specific CTL, but I won't get into that today.

We immunized patients monthly for six months, and we used GM-CSF as an adjuvant, and the primary endpoint of our study obviously is safety. It's a Phase I, but the secondary endpoint is really to see if we can generate immunity.

And when I talk about the generation of immunity, I mean quantitative or semi-quantitative measurements of HER-2/neu specific peptide and protein immunity because our bias is if you're immunizing with peptides, it's only the protein specific responses that may potentially be functional, and looking at that immunity in comparison with other antigens, both positive and negative control antigens.

So looking at, let's say, CD-4 specific immunity in terms of what the tetanus response is, am I even getting to the level of a vaccinated antigen? So trying to bring into the system some idea of where this vaccine works in comparison with other known vaccines.

And in addition, I'll show you we do do anneal immunization with KLH at the beginning of the study to see what our level of vaccination is compared with vaccinating against a foreign antigen.

And our strategy inherently built into a Phase I study is to use multiple assays of analysis. So not only are we looking for specific immune responses against HER-2/neu, but also how many of these assays correlate with each other in terms of predicting that immune response and which assay is most robust and reproducible over time.

What I'd really like to just show you a little bit of data on is our eligibility criteria. We felt very strongly to try to immunize patients that we would potentially want to immunize in terms of a Phase II study, and our shtick is that you immunize patients with a vaccine to protect just like an infectious disease vaccine.

So by definition we had to immunize patients who were at a minimal disease state or had no evidence of disease. Yet we're immunizing against a self-antigen, HER-2/neu.

So the risk-benefit ratio for these patients, if we generated immune responses and could elicit autoimmune toxicity had to be worth their

while. So we looked at Stage III or Stage IV patients whose tumors over expressed the antigen of interest, best ovarian and lung cancer.

These patients were all treated prior to being on study, and they were at a point in their treatment where just observation alone was what they were undertaking. These patients had no evidence of disease or minimal residual disease post therapy, and the mandate to the physicians referring the patients was these patients have to be free or disease and off therapy for six months. So these can't be very unstable patients.

We allowed hormones and radiation therapy, but we pretested everyone before they came into the study with the CMI multi-test, which is a classic test of energy looking at seven different recall antigens, and if the patients were anergic, they weren't allowed onto the study because our conjecture was any toxicity we might see may be related to the development of immune responses, and if the patients couldn't develop immune responses, then it wouldn't really be worth their while as a toxicity study.

And what we found, and this is in parentheses, that the vast majority of patients, all

of the patients that we've enrolled to date -- actually the study is closed -- the performance status on these patients was uniformly greater than 85 percent.

But what I'd like to show you was something that we learned that really blew a bias that I had, and I went into this study thinking most patients with advanced stage cancer were going to be pretty immune incompetent, and basically what we found was in 53 patients who walked in the door -- and this is just a small side study once we made this observation -- when we tested patients with the CMI multi-test where the rules are really not very stringent, you have to have a DTH greater than two millimeters to at least two of seven of the recall antigens to be considered not anergic.

Thirty-six percent of patients didn't respond to anything. So they were anergic. We sent them away. Thirty-four patients, or 64 percent, were anergic and they were eligible and they were enrolled.

We found that patients hated being sent away on a vaccine study. So we said to them, "Okay. Well, prove you're anergic. Two of the antigens that are in the CMI are diphtheria and tetanus. Why

don't you go get a DT? When was your last one done? Come back in two months and we'll test you again and then we'll show you that your immune system isn't functional."

And we were able to do that with ten patients, and basically when these ten patients came back after getting a DT, we found that 90 percent of them responded specifically to diphtheria and tetanus, whereas only one patient continued not to be anergic.

So what we've done is we've enlisted another 15 patients on study who were anergic by these criteria, had no responses to any recall antigens, and we've immunized them, and these are the last patients on the study. So I have none of this data.

And we're going to compare to see whether these patients couldn't be immunized to HER-2/neu, but more importantly, couldn't be immunized to KLH, which is our positive control immunization antigen.

And I throw this in to say that our patients were highly selected for being in pretty good shape with minimal disease, and clearly even though they were advanced stage patients, and many

of our patients had breast cancer and were a Stage IV, clearly they weren't as immune incompetent as I would have assumed.

So what I'd like to end up with and talk about for the rest of my time is assay systems and take you specifically on a tour of a single assay system that we used looking at CD-4 T cell responses in a semi-quantitative fashion.

And I'll tell you that we were hindered a little bit by the fact that most of our patients enrolled in the study were patients with breast cancer, and they could not be leukophoresed in terms of having peripheral lines placed and the challenge of lymphedema, and at this point of starting the study, we didn't feel that we could rightly say to these patients that they should undergo femoral line catheter placement for a leukophoresis for us to get immunologic samples.

So we decided that we were going to take blood from the patients sequentially as they came onto the study, and that we would bleed them 30 days after each vaccine prior to them getting the next vaccine. We would analyze all of the material we got fresh in terms of these assays. So everything that I'm going to show you is on fresh PBMC; and

that we would build into our analysis looking at the reproducibility of these assays over time as a snapshot in patients.

So what I'd like to do is just show you the feasibility of this assay in terms of how much blood you get and exactly how robust an assay like this is.

The immunologic evaluations we're doing in general for T helper responses are looking at modified limiting dilution proliferation assay first described by Mario Geisen and published by Reese in 1993. I think the reference is in your handout.

And we've adapted this assay to split well into cytokines to look for specific T helper responses and cytokine production in a limiting dilution fashion with interferon gamma and IL-5.

We're also looking at the development of antibody on the patients in a very quantitative ELISA. Anyone who's interested in this, I brought some slides with me, but I won't have time to go through them for both peptides and protein, and all of the QA for the large number of patients you need to establish baselines, which are much easier to do serologically.

From a cytotoxic T cell standpoint, we're looking at a limiting dilution analysis based on chromium release, as well as ELISPOT, again, only looking at interferon gamma, but making autologous targets on all the patients using autologous BLCLs and fibroblasts.

And finally, I will talk a little bit about DTH that we do at a distant site using the individual immunizing peptides, looking not only at induration, but also histology, and what I'll do is give you a comparison of this proliferation assay with the DTH assay as an unknown assay compared to the gold standard DTH, which I'm not quit convinced is a gold standard, but it's really the best we have.

This is an example of the assay, and this is an example of the data that we get on the patients and how the actual report looks when it comes out of our group, and Kevin Witham and Kathy Schiffman in the lab have spent a lot of time developing database programs that allow direct downloading of data from our plate readers into the data bank and to have formats like this put up.

This was a modified limiting dilution analysis looking at T cell proliferation as a

functional assay based on looking at 24 replicates of a single E to T ratio against multiple different antigens. It's semi-quantitative.

Basically, you take the mean and three standard deviations of 24 wells of no antigen, and that gives you a cutoff point, and any well above this cutoff point that's positive is positive with 95 percent confidence interval because of the antigen that was supposedly placed in the well, and the error on this assay statistically is 1.5 wells.

So if you have two wells positive, it's what we use to call the assay a bust assay of the no antigen well. It kind of invalidates your assay.

And basically we set up this and plot every single data point, and this is the panoply of antigens that we use. We use about 15 antigens, not only a combination of positive and negative controls, the negative controls being the no antigen and peptides that aren't in the patient's immunizing mix that are similar length and size, but also positive controls, such as PHA and nonspecific mitogen of which you see no dots because it's totally off the area, as well as KLH to which the patients were immunized at the start of the study, and then on another plate a panel of recall

antigens, tetanus, candida, and other antigens that they would have a CD-4 response to endogenously.

And not only do we express this as positive wells here, but also as a stimulation index that's calculated off the mean of a 24 well replicate, not just the mean of positive wells, over the mean of 24 no antigen wells, so that it gives some statistical validity.

So this is the type of data I'm going to be showing to you in terms of reproducibility over time.

The data that I'm going to show you is based on 40 patients, the first 40 patients enrolled in the study, and they get seven blood draws, seven to eight blood draws. We try to do two blood draws pre so we have cells frozen back, sa well as sequential blood draws during the course of all six immunizations.

So that gives you actually the potential for 280 samples. Actually what we got was 218 blood draws, about 180 to 240 cc's each, with 203 assays available for analysis.

In the modified LDA it requires 75 million PBMC, and the mean range of PBMC yield out of these 218 blood draws is 162, with some of the

patients really not having enough cells to even run the assay at a particular time point. So out of the 203 assays that we have, 173, 85 percent, of the blood draws actually yielded enough cells to do the complete assay as I showed you.

In those 203 assays, we had an error rate of three percent of what we called inaccurate assays, and this is data that we don't use, and we describe inaccurate as a control problem.

So if the no antigen wells were greater than two positive wells and no antigen, meaning that there was some type of autoresponse or maybe something was in the media, that is not considered an assay that can be used.

Similarly, if less than 24 wells are stimulated with PHA, meaning that this nonspecific mitogen wasn't generating the response it should, that assay is not used.

So based on those very strict negative and positive controls, only three percent of the assays were not usable. And the number of data points that we achieved out of these 203 assays was 2,242.

So how do you analyze this for reproducibility, sensitivity, and whether this means

anything in real time? And for that we solicited the help of Ted Gooly, a biostatistician at the Fred Hutch. and convinced him to move away from clinical stuff and try to help us talk about immunologic monitoring.

And the first immediate question was: do these positive wells correlate to a stimulation index? And as you increase positive wells, do you increase the stimulation index?

And so what Ted did was Spearman's rank correlation where he took all the data on all the patients at a specific time point, like time zero, to a specific antigen and ran P values, which all were significant, and then he made a rank association, and basically this is a stimulation index against number of positive wells with the rank association not done in 3D, showing you a scattergram that, indeed, the simple question do positive wells correlate to stimulation index, yes, and indeed, the more positive wells you get, the more like your stimulation index is to be elevated.

And basically, once you have half the wells positive, that's when you really start seeing a stimulation index greater than two, which could be consistent with an immunized response.

Now, the reason why we decided to use a semi-quantitative assay is that we went in with the bias that most of the responses that we'd try to be seeing would be extremely low level. So many of the studies that had already been reported before in Tumor Immunology for peptide immunizations, you stimulate with a peptide or immunize with a peptide; you see a stimulation index, maybe three or four.

So we were optimizing looking at responses that were less than stimulation indices of four, and this is what some of the data looks like sequentially. So these are individual patients against one of the peptides in their immunizing mix, and basically these are time points that were taken over six months to show reproducibility.

And the nice thing about this assay is it potentially controls for any background variability by setting the cutoff point of that assay with the no antigen wells of the assay itself.

And what we found was that the assay was really quite reproducibly over time; that patients did, indeed, boost responses. This is stimulation indices, are the square data points, and number of positive wells are the round data points; and that

you could actually see that people developed significant stimulation indices to peptides.

And what I'm not showing you here is also to protein because we don't have protein DTH responses to compare.

But it also points out one of the potential problems of the semi-quantitative assay, and that's once you hit a certain stimulation index or 24 positive wells, you've pretty much lost your ability to enumerate your responses. So you really have to look at the sensitivity of the assay, and basically we hit the sensitivity of the assay.

And so fortunately we have cells that we've been working on since we do blood draws so much and so much blood from the patients that we can develop, and we have developed, quantitative ELISPOT assays to look at three different cytokine secretions of CD-4 T cells to see if we can get a handle on exactly what these precursor frequencies are in real time.

And then to show you this again with not only P-98, which is a HER-2/neu peptide, but also a recall antigen, that again, if you're starting out with a fairly robust, proliferative response with number of positive wells, you're not really going to

be able to detect a boosting and immune response like you could with this peptide.

But it also points out a problem with the stimulation index and the fact that the stimulation index is very variable, probably depending on culture conditions, and indeed, although number of positive wells didn't change over time, this patient did have one data point where she had a big bounce in the stimulation index.

And when you look at, let's say, PHA in these patients, nonspecific mitogen PHA, and if you go back into the literature and look at PHA responses, they're always shown on a log scale like this, and that's because if you look at the stimulation index of individual patients with PHA assessments done at monthly time points, just as part of our assay, stimulation indices really bounce around quite a bit, and if I didn't plot this out on a log scale, you'd say, "Boy, that really is a lot of variability in assay."

So, again, you have to really define the sensitivity and specificity of the assay and also develop a feel for what the noise of the assays are. I would say stimulation index is a good look at very robust responses. There's a lot of background noise

compared to the modified limiting dilution type of analysis, but yet the sensitivity of the modified limiting dilution analysis has kind of reached a peak.

So how does this correlate to real time? And I'd like to show you some data on DTHes. We DTH everyone to their individual immunizing peptides at the end of the study, and basically this is a patient who had a good response to 369-15 MER with a DTH of 17 millimeters, SI of 35, and the DTH was a CD-4 infiltrate.

When we look at how DTH correlates to the peripheral blood SIs we're seeing in over 60 skin tests placed, actually DTHes greater than ten millimeters had a very good correlation with an odds ratio of 11.

If I showed P values for every single time point, five, six, seven, eight, nine, as the DTH induration got larger, the P value would get more significant, but if you look at it as a group, the odds ratio is pretty good at 4.4.

So in our hands, DTH responses correlated very well to peripheral blood T cell responses as measured.

So to conclude, I think the important thing really is to define what the trial endpoint is. For us it was immunity, as well as a comparison of assay systems as an endpoint evaluation, which I hope to have more data to show you in the coming year, to optimize your population based on the endpoint you want to achieve, to use multiple measures of evaluation uniformly, and also to standardize not only your laboratory procedures and controls, and SOPs are things that we use routinely in the lab.

And I'd like to thank specifically my group at the University of Washington: Keith Knutson, Kathy Schiffman, Kevin Witham, Paul Crosby, and Charles Bendock, who are responsible for the clinical trial laboratory.

The peptide vaccine is supplied by Corixa; GM-CSF by Immunex, and I'd like to thank Mac Chiever at Corixa who's been my collaborator in this from the beginning, and Ted Gooly who's continuing working with us on determining the sensitivity and specificity of immunologic monitoring assays.

Thank you.

(Applause.)

DR. SIEGEL: Our next speaker is Dr. Jeff Schlom of the Cancer Institute, who will talk to us about synergy of co-stimulatory molecules in two cell activation.

DR. SCHLOM: May I have the first slide, please? I'm getting signals from up there.

Well, let me just start. One of the major problems in vaccine development is the potency of the T cell immune response, and it is well known that there are many, many ways to try to enhance that immune response.

We have been involved in a series of preclinical studies and clinical trials with a variety of immunogens, recombinant vaccinia virus being one which is replication competent; recombinant Avipox viruses, in particular, ALVCA or recombinant fowl pox, which are replication defective which means that they infect mammalian cells but do not replicate in mammalian cells; peptide; and modified peptide.

Our preclinical studies and now some of our early collaborative clinical trials have shown that it appears that it's a diversified vaccination protocol which gives the optimal immune response, and the work I'm going to talk about not only deals

with vectors or peptides. I think it is quite relevant to whole tumor cell vaccines and dendritic cell based vaccines.

So the major question we set out to ask is: to what limits can one employ co-stimulation to enhance T cell activation?

Now, a little review of co-stimulation. If you have an antigen presenting cell, such as a tumor, which has an antigen and MHC complex, peptide MHC complex, and this interacts with the T cell receptor of a T cell, the outcome of that is going to be anergy and apoptosis because there's no second signal, no co-stimulatory signal.

If you have a professional antigen presenting cell, such as a dendritic cell, monocyte macrophage B cell, you have your antigen MHC T cell receptor signaling, and then you have a second signal, a co-stimulatory molecule to its receptor providing signal, too.

And if you have both signals, you see clonal expansion and other effective functions, cytokine release lysis.

Okay. Now, T cell co-stimulation is a well established phenomenon. It's been shown in

many, many cases in preclinical studies to enhance T cell responses and enhance antitumor immunity.

The mode of delivery for about 95 percent of these studies has been retroviral vectors. We have not used retroviral vectors because of the requirement of drug selection and DNA replication of cells.

There's been some work done with anti-CTLA-4 antibodies. These are antibodies which are really looking at the B-7 co-stimulatory molecule.

We have used, as have others, pox viruses, and we've done this in two ways: making dual gene constructs, so a vaccinia virus or an AVIPOX virus with a tumor antigen like CEA and a co-stimulatory molecule like B-7, both on the same vector; or simply add mixing vaccinia CEA with a vector vaccinia B-7.

Okay. Now, what are the potential advantages and disadvantages of using pox virus vectors to deliver co-stimulatory molecules?

The major advantage of one of the major advantages is rapid infection of the majority of cells. Greater than 90 percent of cells express the co-stimulatory molecule in five hours. So you can envision a tumor, a dendritic cell, whatever. You

put in the pox virus vector and within five hours 90 percent of the cells are expressing the co-stimulatory transgene.

There's no need for cell division or drug selection as with retroviral vectors, and also very important, one can insert multiple genes, multiple co-stimulatory molecule genes or multiple tumor antigen genes. This is something unique to pox virus vectors.

The potential disadvantage, especially with a replication competent virus such as vaccinia, is anti-vector responses, although if you're putting it inside a cell, it may not be a disadvantage but an advantage.

And if you're dealing with a replication defective virus, like AVIPOX or fowl pox, it really shouldn't matter.

The question with co-stimulation is always autoimmunity. How much are you going to stimulate and what are the consequences in terms of antitumor immunity versus autoimmunity?

Before I start showing data, I want to acknowledge the people who carried these studies out. The vast majority of the studies that I'm going to talk to you about were carried out by Dr.

James Hodge and members of his group, Arial Rad and Dr. Matthias Lorenz.

Other studies were carried out by Helen Subzevari and Judith Kanter.

Our collaborators in these studies, some of these studies, were Thereon, Dr. Dennis Panicali, Linda Gritz and Gail Mazzara.

There are two clinical trials ongoing now with an ALVAX CEA B-7 construct, one by Howard Kauffman at Albert Einstein, and one at Fox Chase by Dr. von Mehren.

But I want to talk to you about the basis concept of multiple co-stimulatory molecules. Ninety-eight percent of the literature involved B-7 and the activation of its ligand, CD-28, but indeed, there's a range of co-stimulatory molecules that are now known, and this is just a partial list: ICAM-1 and LFA-3. They have different ligands, and they signal through different mechanisms.

And this is a standard co-stimulation assay. The assay involves an antigen presenting cell, in this case MC-38 murine tumor cells which have no co-stimulatory molecules on them, as I said, virtually all nonhematologic tumors.

Our responder cells unless otherwise mentioned are always naive T cells, and sigma 1 here is Con A. We see the same results with anti-CD-3 or peptide, et cetera.

So that no one loses interest to these studies, we'll use the generic antigen here, Con A, and you can see that you do see some stimulation when you infect your antigen presenting cells with LFA-3, ICAM-1, and the best result is with B-7. You get the best stimulation of your T cells in this rank.

I'm going to show this slide again later on.

This is just a control to show that all of the effects we're seeing can be blocked by the specific antibody to these particular co-stimulatory molecules.

Now, these are all published; the next two slides are published data where we look at an antitumor effect of a vaccinia CEA in the experimental model. Use one injection, and you see a little bit of antitumor effect. If you simply add mix this with a vaccinia virus expressing B-7, you amplify the antitumor effect.

You see the same type of situation with a MUC-1 vaccine, a vaccinia MUC-1. You see an antitumor effect of established lung metastases with three injections of MUC-1, but if you simply have one injection, the first injection with a B-7 molecule involved, you see long-term immunity, and these mice go on to live out their days.

Now, the question I want to dwell on now, and this is the rest of the talk, is: will a triad of co-stimulatory molecules, B-7, ICAM-1, and LFA-3, enhance T cell activation to a new threshold? Now, that is the question.

We chose these, again, because the ligands are different, and the signaling is different, and we have used the term TRICOM to mean -- it's just an acronym for triad of co-stimulatory molecules. So these are vaccines which have a vector which has B-7, ICAM-1, and LFA-3.

So if you see vaccinia TRICOM, it is this so that I don't have to say this over and over again. If you see RVCEA TRICOM, it has four genes in it, CEA and this, and AVIPOX virus is RF CEA TRICOM.

Now, these are the vectors. They all have different promoters, and these are the

controls. Time doesn't allow me to go through all of this, but this is what a recombinant vaccinia TRICOM looks like; recombinant vaccinia CEA TRICOM with the different promoters; and this is recombinant fowl pox CEA TRICOM with the different controls.

The first question we asked is: if we infect cells with a TRICOM vector, will all of the co-stimulatory molecules be expressed on the cell surface? And the answer is yes. These results were obtained five hours after infection of the tumor cell.

So this is the assay that we used. I actually explained it to you before. The antigen presenting cell is the tumor. The responder cell is the naive T cell, and we can use one of any kind of signal 1. Most of the data I'll show you is with Con A, and signal 2 is provided by either a TRICOM vector or a vector containing two co-stimulatory molecules or one B-7, being the current gold standard, and we always use vector controls to show that anything that we're doing is not related to just the vector itself.

This is the data I showed you before, the exact slide, the three co-stimulatory molecules individually activating T cells.

This is the exact same data from the same experiment, but I've added on here the TRICOM, the one with the three co-stimulatory molecules, and you can see there's a great deal more activation.

We asked the question now: can we activate isolated CD-4 cells? Again, ICAM, LFA-3, and B-7 alone; this is the TRICOM, to a much greater threshold than we thought we would see.

This is CD-8 cells, purified CD-8 cells, LFA-3, ICAM, B-7, and the TRICOM, and I want to draw your attention to the low levels of antigen 1, which is more like you would see physiologically. There is essentially nothing going on here with the standard co-stimulatory molecules, and this is what one sees with the TRICOM.

And this is the same data plotted up, but I've added in here a dual gene vector, B-7 and ICAM. You see a little better than either one.

The point I want to make here, that it is not additive. There's clearly something very synergistic going on with having all of these three expressed the same time.

And what we've seen is that the most dramatic differences in stimulation of T cells by TRICOM vectors are observed under conditions of low levels of signal 1 and low APC to T cell ratios. These are the kind of conditions one would see physiologically, and we've seen stimulation of both CD-8 and CD-4 cells.

Now, we also looked at the T cell function in terms of cytokine. These are the various vectors, and you can see in the CD-4 cells we see a great increased production of IL-2, and in the CD-8 cells with the TRICOM, the greatest production is with interferon gamma, and there's a whole range of cytokines. These are studies conducted by Helen Sabzevari.

If you normalize for the reporter gene, you can see here looking that compared to the B-7 or any of the other single ones, the TRICOM stimulates interferon gamma and IL-2 in CD-8 and CD-4 cells far greater than any of the other co-stimulatory molecules.

This is the actual cytokine release profiles, the secretion, and again, you can see B-7, which is the gold standard at this current time, as compared to TRICOM in terms of IL-2 secretion by CD-

4 cells, and interferon gamma secretion by CD-8 T cells.

And now I want to discuss dendritic cells for a second. I wasn't going to talk about this because these studies are so preliminary, but with all of the dendritic cell mavins at this meeting, I thought I would discuss this a little bit.

Dendritic cells express, of course, co-stimulatory molecules, high levels, and high levels of MHC Class I and II. They are the ultimate antigen presenting cell and the most potent simulator of T cells.

We asked the question: can one use a generic APC and infect it with one of these TRICOM recombinant vectors to generate a cell similar to a dendritic cell?

So the current methodology which we followed is taking CD-34 murine bone marrow cells, treating them with GM-CSF and IL-4 for six days to get dendritic cells, and we asked: how would that stack up against taking the same CD-34 bone marrow cells and infecting them with TRICOM for five hours and then seeing what kind of an APC we'd get?

And this is what we see. I'm sorry for the legend here. I'll run you through this.

This is CD-4 cells as your responder cells, and Con A is providing signal 1. If you take CD-34 bone marrow cells, you see this type of activation of T cells.

If you make dendritic cells from these, you see this. So I guess this is the zebra and this is the crocodile.

If you take these same CD-34 cells and infect them with TRICOM, you see this: not quite as good, but in the same range.

If you use CD-8s as your target, these are your CD-34 bone marrow cells. These are these cells treated for six days with GM-CSF and IL-4. These are your dendritic cells, and these are these same CD-34 cells treated for five hours with TRICOM vector: a little better.

So you can see a potential use for this right up front.

The next question we asked, and we didn't think this experiment would work: can one design a better APC than a dendritic cell?

You have CD-34 bone marrow cells. You treat them with GM-CSF and IL-4 to obtain the

dendritic cells. We then ask if one takes these dendritic cells and treats them with TRICOM, would you get some better cell.

This is the exact same data I showed you on the previous slide. This is the CD-34 cell. This is the dendritic cell. This is the dendritic cell transfected with TRICOM. This is CD-4 as the responder cell. These are CD-8 responder cells. This is the CD-34 bone marrow cells, the CD-34 cells treated with GM-CSF and IL-4 for six days, your dendritic cells, and this is not chopped liver. This is 50,000 counts.

This is this dendritic cell then treated with TRICOM.

The next question we wanted to ask was, going back to recombinant vaccines, all the work that we've shown to date has been using naive T cells as responder cells, and there's a little question in the literature as whether you really need co-stimulation for memory cells. If you have a memory cell, do you have to co-stimulate or co-stimulate doesn't matter?

So we looked at both naive T cells, T cells for mice immunized with CEA, and also established cell lines, and we've done all of these

types of ways to deliver signal. It really doesn't matter.

This is just a preliminary piece of data. These studies are ongoing where we take C-57 mice; we vaccinate them one time with ten to the seventh plaque forming units, one injection of CEA or CEA TICOM.

We waited for 100 days, and then we challenged them with tumors expressing CEA, a large dose of tumors, and you can see there's clearly induction of immunologic memory.

And we looked then to see what about proliferation of T cells in terms of looking at these mice splenocytes, and again, you can see that the TRICOM gives you a much more robust response than the standard vaccinia CEA, but these are just the premises for this.

The question we wanted to ask is: can you use these type of vectors and triple co-stimulatory molecules to stimulate memory cells?

And what we did was we took C-57 black mice. We injected them with Avipox vector and waited 40 days and took out the T cells, and those are the CEA immune T cells. We also have naive T cells, control.

And here we see the antigen presenting cell is a CEA positive tumor. This is a CEA negative tumor, with these various co-stimulatory molecules in them, and what we can see here is that we get very, very good stimulation of memory cells, much better than we would see with B-7.

There is something here. It's just that the scale is so large that it's reduced, but there is some stimulation here, but much more stimulation with TRICOM.

We then asked: can we stimulate established cell lines, so-called defector cells? And again, here we have an APC, a tumor cell, and then we take cells and this is a tumor cell infected here with a fowl pox vector expressing these various genes.

And we asked: can we stimulate an established T cell line directed against CEA? Well, if you just put fowl pox CEA in there, you get no stimulation because there's no second signal on your antigen presenting cell, on your tumor.

You put in B-7. You get better stimulation as expected, but you still get even better stimulation with this TRICOM vector. So,

again, we can stimulate now naive T cells, memory T cells, and affecter T cells.

So in closing I want to mention that the classical notion of post stimulation where you have two signals, hence co-stimulation, is now perhaps we're looking at a different mechanism here where we're dealing with four signals, four different ligands, and four different signal transduction processes in some of type of hyper stimulation.

I just want to touch on at the very end -- I'm going to show no more data -- that these are some potential uses for these vectors, for these constructs.

The first is vector based vaccines. One can take ALVAC recombinant and put a tumor antigen in them with the TRICOM as I've shown you.

The other is the infection of whole tumor cell vaccines. There's really no reason why the generic -- no tumor antigen here -- but the generic triple post stimulatory vector cannot be put into carcinoma or melanoma cells either in culture for five hours or direct injection at the tumor site.

Infection of dendritic cells, either peptide pulsed dendritic cells or dendritic cells

infected with a vector to enhance the potential of T cell post stimulation by a dendritic.

And finally, the simple in vitro activation of CD-8s or CD-4 cells by using antigen presenting cells infected with these TRICOM vectors.

So to what limits can one employ co-stimulation to enhance T cell activation? My answer is, and this is just a hypothesis at this point because we need much more data, that new levels of co-stimulation can now be achieved with recombinant vectors containing three co-stimulatory molecules to stimulate T cells to a new threshold of activity.

Thank you for your attention.

(Applause.)

DR. SIEGEL: Thank you very much.

Our next talk is from Jay Berzofsky at the Cancer Institute, who will discuss his work regarding optimization of antigen specific T cell responses using epitope enhancement.

DR. BERZOFSKY: I'd like to thank all of the organizers for inviting me, and in the interest of time go to the first slide.

I'm going to talk about optimization of antigen specific T cell responses for antitumor and antiviral activity. This is the work of a large

number of collaborators, as you can see listed here, and I'll try to mention individual names as I go along.

And I'd like to talk about three issues or points. One is that CTL avidity is important for efficacy and clearing viral infections and may be so for tumor immunotherapy, as well.

Second, that natural viral and tumor CTL epitopes may not have optimal affinity for MHC molecules. Can these epitopes be enhanced to produce more potent engineering vaccines and how can we do that?

And third, can mutations in P-53 in rats found in tumors service tumor antigens to evoke CTL and kill tumor cells, and can we apply these principles?

So by avidity what I mean is sensitivity to very low doses of very low densities of peptide MHC complex, and I don't know if you can see this red curve here with the amount of light on the screen, but if you look at a dose response curve for antigen on a log scale here for recognition by CTL, Martha Alexander Miller was able to raise CTL specific for the same peptide MHC complex, but with very different uveitides. The CTL line on the far

left in red sees antigen at more than two logs lower concentration than the lower avidity CTL line shown in yellow on the right, and there's some intermediate ones in the middle.

Now, in data I won't have time to show, she found that, in fact, the high and low avidity cells would kill targets infected with recombinant vaccinia expressing the antigen. In this case it's HIV GP-160.

And so we asked whether these would be equally efficacious in clearing virus in vivo, and of course, we couldn't use HIV in mice. We used the recombinant vaccine from Pedero and Bernie Moss, expressing GP-160.

And to test this, she adoptive transferred these different lines into Skid mice that have no T or B cells of their own and infected those mice with the recombinant vaccinia and asked what level of virus was found several days later, and you can see this is on plaque forming units of virus per gram of tissue on a log scale.

The white bar are the Skid mice that received no CTL adoptively transferred, and you see that the low avidity CTL transferred either the red or the yellow, two different low avidity lines, had

little or no effect on the level of virus, whereas the high avidity line shown in green gave us more than three log reduction in virus PFU, and this is reproducible with multiple independently derived high and low avidity lines.

So this indicates that the quality of the CTL is at least as important as the quantity of the CTL in clearing virus, and that may be true for tumors as well, and so this may have important implications for adoptive immunotherapy, for example, selecting high avidity TILs rather than TILs grown nonspecifically with cytokines and, for example, trying to develop vaccines that would selectively expand high avidity CTL.

Okay. The second point that I wanted to discuss is trying to enhance the immunogenicity of epitopes. Viral and tumor epitopes are not necessarily the best immunogens. If anything, they may have evolved to escape the immune system. So can we make them better?

And the idea is illustrated here in this crystal structure of a peptide bound to a Class I molecule from Ian Wilson's lab in which the peptide is shown in blue, and we're looking at side chains

in pink of amino acids of the MHC molecule that are interacting with the peptide.

The view that we're looking at is what we think is the view that the T cell receptor would see looking down on the surface of the peptide MHC complex, and the idea is that if we can modify the side chains of amino acids that interact with the MHC molecule in such a way as to improve the affinity of the MHC molecule without changing this surface that's pointing outward toward us, that the T cell receptor sees, then we may have a more potent immunogen that will still induce T cells that will see the natural peptide MHC complex since if it doesn't see that, it's not very useful.

And so we applied this in several cases both for Class I and Class II. The first case I'll just illustrate briefly. It's based on work that we did actually about five or six years ago, done by Toshitaka Akatsuka in our lab in collaboration with Henning Binkey in Ron Jermain's lab in which we found that we could enhance he affinity of a helper epitope shown here in green from the HIV envelope by replacing this positively charged E-glutamic acid -- sorry -- negatively charged E-glutamic acid with an uncharged alanine A shown here in blue.

And so we asked whether this would improve he efficacy of this helper epitope for inducing CTL against the CTL epitope from the HIV envelope shown in yellow that we had also characterized in our lab.

When the CTL epitope itself was not modified, we were only modifying the helper epitope that we attached to that in this synthetic vaccine construct, and this is work by Jeff Ailers in the lab. Indeed, that was the case.

You can see in the open circles the CTL induced by the original vaccine construct and in the triangles the CTL induced by this improved second generation construct, and it requires 33-fold fewer effector cells in this effector to target ratio titration to get the same level of lysis when we immunize with this improved vaccine.

So that means there are 33-fold more lytic units if you compare these curves horizontally, and in data I won't have time to show Jeff did genetic experiments to compare strains of mice that have the same Class I molecule to present the CTL epitope, but different Class I molecule -- Class II molecule, rather, presenting the helper epitope to prove that, in fact, the mechanism of

this was, indeed, Class II MHC length and due to the higher affinity for the Class II MHC molecule.

So this demonstrates two points. One is that helper T cells are very important; CD-4 helper T cells are very important for inducing a Class I CD-8 cytotoxic T cell response. So that by just improving the level of Class II restricted help we can get a big increase in Class I restricted CTL even though we haven't tampered with the CTL epitope at all.

And secondly, this is proof of principle of this approach we call epitope enhancement for trying to improve vaccines, but this was done with the Class II molecule, and it was done in the mouse, and we wanted to know if we could do the same thing with the Class I molecule in a particular one that came from humans.

And so I'll now tell you about a more recent study done by Pable Sarobe in the lab with a number of collaborators listed here that was just published in JCI trying to improve the binding to HLA-A2 of an epitope that we had identified earlier in the hepatitis C virus core protein that binds to A2 with an affinity that's adequate for inducing cytotoxic t cells in patients infected with the

virus, but was still modest affinity and had room for improvement.

And this is the wild-type peptide at the top. Dave Pendleton in the lab made it the large series of substituted peptides shown here, and Pablo tested all of these both for binding to HLA-A2, which I won't have time to show you, and for recognition by human cytotoxic T cells from an HCV infected patient that he had raised, and you can see this is a dose response curve for some of the peptides, the alanine substituted peptides. The wild-type peptide is shown in green in the middle.

Many of the substitutions reduced activity, but for two of the substitutions the dose response curve was shifted to the left, i.e., we had about a tenfold enhancement in potency, but what we really wanted to know was would these be more immunogenic in vivo.

And before going into some kind of human clinical trial, we wanted to use an animal model and were fortunate to have HLA-A2 transgenic mice from our collaborator Vic Englehart that Pablo immunized, and here we're looking at the response to either immunization with the wild-type peptide shown in green or several of these modified peptides that had

higher affinity, and one of them in particular, the 8-alanine substituted peptide, was more immunogenic in the A2 transgenic mice.

So we wanted to know if the CTO we induced against this 8-alanine substituted peptide were of as good quality as those induced against the wild-type peptide for killing of targets expressing the wild-type peptide.

And that's tested here where we compared a CTL line raised against the wild-type peptide and tested on targets with the wild-type peptide shown here in a dose response curve with a CTL line raised against the enhanced peptide with the 8-alanine substitution, but tested in vitro on the wild-type peptide shown in red.

And you can see that this actually has several logs higher avidity than the CTR raised against the wild-type peptide itself. So we had CTR that were increased not only in quantity, but also in quality by this approach.

So we conclude with regard to this approach of epitope enhancement that we've been working on now for about six or seven years that natural epitopes are not always optimal, but can be enhanced by sequence modifications to increase the

binding to murine or human Class I and Class II MHC molecules.

A selected subset of these modified peptides retain recognition by T cell specific for the natural epitope and are substantially more immunogenic for inducing helper T cells or CTL in vivo.

And I'd like to point out that these enhanced epitopes can make more potent vaccines whether they're used as synthetic peptides, as I illustrated, or incorporated as sequence modifications in the gene for the whole protein and used in recombinant protein or in naked DNA vaccines or in recombinant viral vector vaccines or even in live attenuated viral vaccines, where one can cite directed mutants in the virus.

So this approach of epitope enhancement is certainly not limited to peptide vaccines, but could be applied to any type of vaccine construct.

Okay. Now, the third area is to talk about the use of mutant tumor suppressor genes and oncogene products as targets, potential tumor antigen targets, for immunotherapy, and the idea was that single point mutations in P-53 that occur commonly in tumors or RAS that occur commonly in

tumors or others like that might create neoantigenic determinants that could be recognized by the immune system as distinguishing markers present uniquely in the tumor and not in the normal tissue in which the mutation did not occur, and we might be able to raise cytotoxic T cells against these by immunizing with short synthetic peptides just surrounding the mutation so that we would not induce T cells that would see the wild-type protein that's present in the normal cells.

And for the sake of time, I'll skip over the in vitro studies that are published and go directly to an immunotherapy study in mice. this was done by Dimetri Gabrilovich in Dave Carbone's lab in collaboration with us, and you can see that here we're immunizing with peptide post dendritic cells, which is an approach that we had used first again about six or seven years ago with HIV peptides to induce high levels of CTL.

And here you can see complete inhibition of tumor growth by multiple peptide post dendritic cell immunizations compared to either a single immunization or no immunizations.

Similar results have been obtained in other labs. For example, this study from Mike

Lotze's lab looking at the Meth. A sarcoma which has a P-53 mutation, immunizing with dendritic cells post with a mutant peptide, you can again inhibit the growth or cause regression of established tumors.

The other study I just showed you I should have pointed out was also an established tumor before we started immunizing.

In contrast, you can see the dendritic cells post with the wild-type peptide or post with no peptide had no effect on tumor growth.

So we have been involved in a Phase I clinical trial that is done with a large number of collaborators, with Chuck Smith and David Contoise in our lab; with Dave Carbone and John Menna in Dallas and Vanderbilt; and with Mike Kelly and a large number of collaborators working with him in the Medicine Branch here at NCI; and with a lot of help from other people in other parts of NCI, Morris Kelsey and Jay Greenblatt and their co-workers, and a number of others.

And I'll just show you one example here of specificity that we can induce in some cancer patients with P-53 mutations, first of all.

This is a patient who had on CTL specific for his mutation prior to immunization, but at 11 weeks and 46 weeks after starting of immunization, here we're looking at level of gamma interferon response. You can see that the response to the mutant P-53 peptide shown in the solid bars has a magnitude that is a substantial fraction of the magnitude we see to whole flu virus, which has multiple epitopes, and here we're just looking at one epitope. So for single epitope that's quite a substantial response.

Whereas there's no response at all -- you can't even see the bars here -- to the wild-type peptide. So this is exquisitely specific for the mutation and, therefore, would not see normal cells at all, which is the goal we were trying to achieve.

Now, we wanted to know if these would kill tumor cells expressing mutant P-53, and in this trial we weren't able to get autologous tumor to test, but fortunately we now have finally been about to obtain data along those lines in this very recent study done by Sarah Gur and Hong Kung in Samir Khlief's group working with our lab, and this is a collaboration with Bernie Fox and Walter Urba from the West Coast, who have immunized in this case a

breast cancer patient with breast cancer cells that were transfected with the co-stimulatory molecule B-7 that you were just hearing about from Jeff Schlom.

And these induced an immune response, and we asked whether that immune response included a response that was specific for a mutant P-53 that was present in this breast cancer tumor.

And so you can see here that, indeed, looking at autologous targets in either the absence or presence of the mutant P-53 peptide, we get CTL that are very specific for the mutant P-53. So we asked whether these P-53 specific CTL would kill the tumor cells.

And so we expanded CTL now with the mutant peptide, and as a control, expanded CTL from the same post immunization PBMC with flu, and you can see that the CTL expanded with the mutant P-53 peptide will kill targets, kill the tumor cells as targets, whereas those expanded with the control antigen do not.

So these are mutant P-53 specific CTL that are killing tumor cells expressing that mutation.

Now, turning to RAS, for mutant RAS we have a more limited number of mutations occurring most commonly at CODON 12 as listed here, and these have been studied by Chuck Smith in our lab for binding to HLA molecules, and he found that these bind with a moderate affinity to HLA-A2, but an affinity in the range that people have seen for other antigens that are recognized by human T cells.

And, in fact, you can see that this segment containing the mutation has a classic HLA-A2 binding motif with a leucine at position 2 and a valine at the C terminus, and we've immunized patients as part of this trial with the various mutant RAS peptides corresponding to the mutation in their tumor.

Here's an example of one patient who made a CTL response to RAS-12 CIS, a peptide we call PR-18, and you can see quite a high specific lysis compared to controlled targets with no peptide.

And, again, in this trial we were not able to get autologous tumor to test this target, but there are now two publications in the literature that show that, in fact, these mutant RAS peptides are presented on tumor cells and can be the targets of CTL.

One of these is from Abrams, et al., a study from Jeff Schlom's lab done in collaboration with Samir Khlief in Medicine Branch, and they were able to induce CTL that will kill colon carcinoma cells expressing HLA-A2 and this RAS-12 mutant peptide shown here, whereas the CTL specific for MART-1 as a control do not.

And Gjertsen, et al., from Norway have shown similarly that this same RAS-12 valine mutant peptide is also presented by HLA-B35, and CTL raised against this will kill autologous tumor cells expressing B-35, as well as allogeneic tumor cells that share HLA-B35 but not B-35 negative tumor cells.

So you can see that these mutant RAS peptides are presented with at least two different human Class I molecules on tumor cells and can make those tumor cells the targets for lysis by CTL that are raised by vaccines.

So in conclusion and in summary, high avidity CTL are more effective at clearing viral infection than low avidity CTL, and the same may apply to adoptive immunotherapy of cancer and may be important for designing vaccines both for viruses and for cancer.

Epitope enhancement by sequence modification allows production of more potent vaccines by increasing affinity of peptides for Class I or Class II MHC molecules, and you saw proof of principle of this.

In murine models, vaccines consisting of dendritic cells presenting mutant P-53 peptides can elicit specific CTL and treat established tumors, and in humans, mutations in P-53 and RAS found in human tumors can serve as tumor as antigens. Vaccines specific for these mutations can elicit human CTL that kill human tumors expressing the corresponding mutant protein.

And so we have a case now where we can try to apply this approach of epitope enhancement, which we're doing now in these cases, to try to enhance the immunogenicity of these peptides, and we can try to use measures to elicit higher avidity CTL to improve the efficacy of cancer immunotherapy as we are trying to do for viral therapy as well.

And you saw an example of this that Steve Rosenberg presented earlier from some recent data of their lab where they've been able to apply epitope enhancement to a GP-100 peptide.

So I think that these same general principles that have been developed originally for viral antigens will apply to tumor antigens and can perhaps produce more effective vaccines.

Thank you.

(Applause.)

DR. SZNOL: Thanks, Jay.

I'd like to introduce the next speaker, Dr. Nicholas Restifo from the Surgery Branch to talk about cancer therapy using a self-replicating RNA vaccine.

Nick.

DR. RESTIFO: Thank you, Mario, and thank you, Raj, for inviting me to this meeting.

I'd like to talk to you today about the work that we're doing in trying to develop mouse models that will help us sort through this dizzying array of possibilities that we have for cancer vaccines for use in the clinic.

Now, it's my belief and since I started in on these efforts almost ten years ago that mouse models can be predictive for the clinic. Some mouse models, however, may be more predictive than others, and I think the quality, the ability of a mouse

model to predict what's going to happen in the clinic requires a certain number of things.

First of all, I think a focus on treatment of tumors rather than their prevention. It's a rare chance that we have of being able to guess a patient that's going to develop a cancer, and much more of a common occurrence where we're trying to therapeutically vaccinate a patient.

The other thing is to choose the right antigens to study, and so I'm going to focus on our recent efforts in this direction and focus my comments on developing vaccines in a mouse model where we use self-antigens, melanocyte differentiation antigens.

You've heard earlier from Dr. Rosenberg efforts at cloning the antigens that are recognized by antitumor T lymphocytes. So I won't spend much time on that.

These antigens are generally melanocyte site differentiation antigens, and interestingly, many of these antigens are involved in the generation of the actual pigment, melanin. They're enzymes, such as TRP-2, further down the line GP-100, TRP-1, and tyrosinase that are involved in the actual pigment formation.

Now, there are a lot of reasons why we think these are good antigens, but there's one reason why they're not, and that is tolerance, the problem of immune tolerance against nonmutated self-antigens.

So to more directly look at this question of tolerance, we've done a number of things. Specifically, we have cloned the homologs of the human melanoma antigens in the mouse. So these enzymes have remarkable homology between the mouse and the human.

And by using the actual mouse antigens, we're able to, I think, more accurately model the situation of what we're trying to accomplish in patients.

Now, a number of things have been done. One of them is to identify, now, the genes in the mouse and to study the knockouts in some cases of these genes, where we can study situations where these antigens are present and when they're absent in the mouse.

Now, here's a listing of these antigens: tyrosinase, TRP-1, TRP-2, GP-100, MART-1, listed together with the mouse loci corresponding to these

genes and alternative names which you may have read in the literature.

This is a photo of some mice which are knocked out for GP-100, which have a variable phenotype, some of which have this nearly albino appearance, and for TRP-1, which have this more -- which Chris Tulukien who's heading this effort calls a capuccino phenotype.

(Laughter.)

DR. RESTIFO: We have a lot of coffee going around in the lab.

So this is very interesting. These are really on a C-57 black 6 background and he's breeding those.

And so these mice are going to give us some interesting insights, I think, into the development of or the issues of tolerance in these mice.

Now, using vectors constructed with the mouse homologs of these melanocyte differentiation antigens, we've attempted to do a number of things in normal, nonmanipulated C-57 black mice. One of them was to generate CTL, which we've been able to do against mouse GP-100. I won't go into the details of this. It was recently published in

Journal of Experimental Medicine, in work done by Villum Overwick in the lab, where he was able to generate CTL.

Another one of these, the vaccinia viruses that he built in collaboration with Bernie Moss, the murine TRP-1 had a different effect, and it induced a profound co-color changes in the C-57 black 6 mice where you see this vitiligo-like syndrome, sometimes in a dermatomal distribution, but you can see a real heterogeneity in the induction of vitiligo similar to what we see in the patients.

Now, the fur on these mice is absolutely snow white, as you can see, and reminds us a lot of some of the patients who successfully respond to Interleukin-2. Here you see this patch of light hair and light skin.

And also, in patients, as mentioned by Dr. Rosenberg earlier, we see inflammatory regions, inflammatory areas surrounding either moles or regressing melanoma lesions. Here we see a vitiligo, patch of vitiligo around those.

Now, so what I'd like to focus my comments on is what these animal models predict for the future of recombinant and synthetic anti-cancer

vaccines. I mentioned the effectiveness of targeting melanocyte differentiation antigens.

Those vitiligo mice are absolutely protected from challenge with B-16. The GP-100 CTL I showed you earlier can be adoptively transferred to mice bearing established B-16 pulmonary E. mets. (phonetic), and they can be used to treat. So we feel that these are valid antigens for targets, which we can use our vectors to target.

But specifically now, in terms of the form of the antigen, Jay Berzofsky, I think, was way out in front in describing the use of these so-called anchor fixed antigens, that is, antigens whose ability to bind to MHC molecules can be improved by altering their amino acid composition.

We have also explored the efficacy of endoplasmic reticulum insertion signal sequences both in recombinant and synthetic immunogens, that is, putting the right antigen in the right intracellular compartment, which for Class I is the endoplasmic reticulum, and finally, the important role for self-replicating nucleic acids.

Now, I'm just going to briefly touch on our efforts at anchor fixing, that is, specifically to increase the binding of the peptide to the

restricting Class I molecule without inhibiting its ability to bind to the T cell receptor that recognizes it.

Using these approaches, we found a naturally occurring anchor fixed epitope, the mouse versus the human form of GP-100 in mice, in C-57 black 6 mice. The GP-100, 25 to 33, in the mouse differs in three amino acids from the human, but that difference increases the binding to D of B significantly. The human binds much better, and that's all been reported in HR Medicine, and that seems to be an excellent way of breaking tolerance against GP-100.

We've recently reported in HR Medicine our efforts at altering the second position of the 209 epitope, which significantly increases its binding to HLA-A2 and some other examples.

Now, using these altered peptides, either the 209 or the 209-2M, in the form of a recombinant fowl pox virus with a form of modified GP-100, and this work done in collaboration with the Thereon Corporation, something we hope to get into patients, we've modified the human GP-100 at two positions, the 209 at the 2M position and the 280 at the 9V position.

But you can see here in a transgenic mouse situation immunization with the modified form of the recombinant fowl pox virus significantly increases its ability to generate CTL not only against the modified form of the 209 epitope, but against the wild-type form of the 209 epitope as well.

I'd like to comment now about the introduction of antigens into the endoplastic reticulum. This is a diagram showing the transport of antigens from the site, as all three of the TAP transporters into the ER, but it's also possible to by pass these TAP transporters by using ER insertion signal sequences, and this work was done in collaboration with John Udall and Jack Bennick of the NAIAID.

What we've done is we've used an insertion signal sequence from the E-3-19K protein of the adenovirus and attached that to the modified form of 209-2M.

Now, used in a mini-gene form is another construct that we're very excited about. We plan on exploring the use of this recombinant pox virus, specifically with emphasis on the fowl pox virus, in

patients with melanoma, again, another construct built for us by the folks at Thereon.

Using the ES sequence together with the GP-100, the 25 to 33 epitope, again, you can see that we can do things with the ES modified peptide that we can't do with the native peptide.

So, again, you can see there are many combinations of using anchor fixed peptides alone or in combination with ER insertion signal sequences.

Now, the final part of my talk, I'd like to comment about the different vectors that we have available to us. Using these, the mouse homologs of the human differentiation antigens, we've constructed a number of different recombinant viruses, including vaccinia viruses, fowl pox viruses, their nonreplicating cousins, transfectant, so-called transfectant Influenza A viruses, transfectant because you add an additional really chromosome into the flu virus, and recombinant adenoviruses.

Now, we've also begun to look at other forms of the antigen, including naked DNA in a number of forms, as well as Baculovirus proteins, and you've heard about the synthetic peptide efforts.

Now, we've heard also quite a bit about dendritic cells, one of the cells we think is central. This is where we're trying to get our various vaccines, our recombinant and synthetic anti-cancer vaccines.

The evidence that we have that dendritic cells are the active cell in the use of recombinant pox virus comes from evidence where we were trying to optimize the promoters that we were using in our recombinant pox viruses.

We used promoters. Now, the number here is the relative amount of our model antigen, beta-GAL that was produced if you use the P-7.5 promoter as one. We had promoters that could produce 300 times more beta-GAL than the normal 7.5 promoters. These are synthetic promoters in vaccinia virus, but those weren't the most powerful promoters in treating tumors.

It was the early promoters, even if they were relatively weak compared to the late promoters, and that had to do with their activity in dendritic cells.

In the use of pox viruses though, I think in some ways we're a victim of our own success. Vaccinia virus was used in the successful

eradication of smallpox essentially from the face of the world. It's only around now in a couple of labs, probably more labs than we think perhaps, but the vaccinia virus eradication program, the eradication of smallpox was a profound success.

But people remember that vaccination even if they got it 50 or 60 years ago, and the neutralizing antibodies, the preexisting antibodies against vaccinia virus survive in our patient population. Any patients older than about 30 generally have these neutralizing titers.

Now, the titers against fowl pox virus, except maybe in a few chicken farmers, are relatively low, and so we have focused our efforts on the recombinant vaccines on the fowl pox virus.

But the general problem of vector associated proteins is a real one. Vaccinia virus expresses over 200 genes, and there's going to be immunity that's going to prevent the repeated use of these vectors.

And you can see that Influenza A, a relatively small virus with eight genes and eight gene products, ten -- sorry -- gene products, is smaller, but there's still vector associated protein.

So we've aimed our sights at naked DNA as an immunogen. Now, we believe that naked DNA works in much the same way. In a collaboration with Ron Jermain and Angel Porgidore, we've explored exactly how these plasmids work, and we feel and have published recently that bone marrow derived antigen presenting cells are the likely targets for these nucleic acid vaccines.

But there may be some role for the regurgitation of antigen or even the direct expression of antigens in transfected myocytes, for example, after intramuscular injection.

We have in collaboration with folks at VICAL initiated a clinical trial where we use a modified form of the human GP-100 with the 209-2M modification, the 280-9V modifications in patients, and there are a lot of things you can do to optimize a vector.

You can remove the three prime and five prime untranslated regions. You can optimize the promoter. You can introduce nuclear processing signals to make these vectors better, controlling the polyadenylation sequence.

But we may have to make these vectors even better. There are two major problems that we

see with the use of these vectors in the current technology. One is the efficiency with which the vectors get into the appropriate cells, antigen presenting cells. That's the first one.

But the second one is the power. Do these vectors have the kind of power that the viral vectors have? And they clearly don't if you compare them with the recombinant viruses that we're using in preclinical studies.

So what we've done is we've developed a replicase-based vector. Now, this is an attempt to make DNA more similar to a viral infection. What we've done is -- and this is all work really spearheaded by Han Ying in the lab and how he's recently been joined by Wolfgang Leitner -- where a CMV promoter is placed in front of a replicase gene derived from alpha viruses, either Sembis virus or Semliki Forest virus.

That replicase gene is then able to copy an RNA, a positive stranded RNA into a negative stranded RNA and back again, and this can lead to massive amplification of the RNA.

You can also use just an RNA form of this virus, and in that case you don't need a CMV promoter where you insert the replicase complex.

We've constructed all of the appropriate controls for these vectors, and when we measure their ability to induce CTL, we see something that we don't see with conventional plasma DNA vectors, the ability to induce specific CTL at extremely low concentrations of RNA shown on this slide.

So in submicrogram levels of RNA, we can induce a specific antigen recognition of these tumor targets or peptide pulsed tumor targets.

Furthermore, something that we don't see with conventional plasma DNA, we can see treatment of established tumor with an increase in survival, and not shown here a decrease in the number of pulmonary mets.

We developed this replicase-based plasmid, RNA and DNA immunogens, with the goal of increasing the antigen production, but when we measured the amount of antigen that was actually produced, it was just a little more than twofold more antigen compared with a conventional optimized plasmid, just a little better. So we had to look for other reasons why it was better.

When we actually measured the production, however, we saw that the cells were almost uniformly dying quantitatively after this,

and this death was apoptotic, and you could inhibit that death or at least delay it by the addition of CAS-based inhibitors.

CAS-based are enzymes that are involved in signal transduction in the mediation of apoptotic death.

Now, we've heard a lot yesterday about apoptotic cells and how they may feed into dendritic cells, and we think this might be a clue to why these vectors work better, but there are clearly a number of potential effector mechanisms why the replicase-based nucleic acid vaccines are more effective.

And so what do animal models predict for the future of recombinant and synthetic anti-cancer vaccines? They predict the effectiveness of targeting melanocyte differentiation antigens, I think a point that's been borne out in the clinical studies.

They predict the use of anchor fixed antigens that have increased binding to MHC.

They predict the efficacy of using endoplasmic reticular insertion signal sequences to put the right antigen in the right intracellular compartment.

And they predict an important role for the use of self-replicating nucleic acid vaccines.

Now, the cell might be the basic unit of life, but the bench researcher is the basic unit of getting these results, and these are the people that did the work: Mark Theore, Villum Overwick, Chris Tulukien and Tanir Alweiss, Carie Ervine, Han Ying, and Wolfgang Leitner, and Debbie Sermon, and we've recently been joined by Peter Emtage.

And so I thank you for your attention.

(Applause.)

DR. SZNOL: Thank you.

The last speaker for this session I'd like to introduce, Dr. Bernie Fox from the Earle Chiles Cancer Research Center in Portland, Oregon.

Bernie.

DR. FOX: You did a great job. Now I know why I've got gray hair. It's vitiligo.

(Laughter.)

DR. FOX: Too much immunology, too many adjuvants.

What I'd like to do this afternoon is tell you a story that's developed in my lab over the last two years, and while a big focus of this meeting has been on the antigens and on the vaccines

and trying to characterize those, the focus of our efforts have really been to try and understand the effector mechanisms that are involved.

And it's kind of nice to follow Nick because I think a lot of what we're looking at is tolerance, and I hope that I can tell you by the end of this talk that we think tolerance is also involved in this mechanism, but it's a different form of tolerance at least in the early stages, and it's the form of immune deviation, where we think it's the development of an ineffective or nondestructive immune response that develops in response to vaccination that sometimes biases the immune response away from a therapeutic response.

If I can have the first slide.

So the goal of my laboratory has really been to use adoptive immunotherapy as an approach to identify the cells that mediate tumor regression with the idea being that you could then induce these cells in vivo without having to do adoptive transfer.

The model that we've used is a model that C.U. Shu developed while in Steve Rosenberg's lab back in 1985, and it involves vaccinating animals with a tumor and then seven to ten days

later removing the draining lymph node cells that drain that tumor vaccine, and so you showed initially that if you stimulate those cells with tumor, you then can draw -- and provide low doses of or high doses of IL-2 and then subsequently low doses of IL-2 -- that you can generate cells that were therapeutic and would recognize that tumor specifically in vitro and make cytokines.

And the specificity was really conferred by the vaccine, and so if you took lymph node cells that drained a vaccine and stimulated them with anti-CD-3 or subsequently with super antigens, the T cells that come out are exquisitely specific for the tumor that primed their generation initially in vivo.

And this all works fine with weakly or strong immunogenic tumors, but falls apart when you use tumors that are more poorly or nonimmunogenic, and that I'll define as an animal that's been vaccinated with tumor, with an irradiated tumor, and you come back and challenge 14 days later with a minimal tumor dose. If that tumor grows progressively, that's a nonimmunogenic tumor or poorly immunogenic tumor.

So when you do that in a poorly immunogenic setting, you had to do something else because the lymph node cells would never work, and so what we did initially in collaboration with Fred Chang and C.U. Shu and Windy Wahl and Gary Nable and Greg Plautz who's here, we looked at an aloe modification of that tumor vaccine and showed that if you aloe modified the tumor, that the lymph node cells that came out once you stimulated them with anti-CD-3 and expanded them in lotuses of IL-2, they were now therapeutic, and that was the basis for a clinical trial that we've actually just finished.

So in background, the tumor we're going to use in these studies is the D-5. It's a subclone of B-16-BL6. It's poorly immunogenic in that immunization fails to protect T cells from D-5. Tumor vaccine draining lymph nodes are not therapeutic.

However, when we went and looked at them, we found out that those lymph node cells that are nontherapeutic do down-regulate L-selectin, and that's important because down-regulation of L-selectin is a well established marker for recently activated T cells and memory T cells.

And back in 1996, Kagamu, working in C.U. Shu's lab, demonstrated that L-selectin low tumor vaccine draining lymph nodes were enriched for therapeutic T cells in a weakly immunogenic tumor model.

So we went ahead and asked the question: will these L-selectin low cells in the D-5 vaccine model mediate tumor regression if we activate them and adoptively transfer them?

And the model we used, again, was we vaccinated animals and seven to eight days later removed the lymph node cells, used the Milton E-beads to separate the L-selectin low cells out from there over a column so that you have total cells here with a small percentage of cells expressing low levels of L-selectin. This is looking at L-selectin expression by FACS.

And you can separate those cells using the beads into either low or high populations. We went ahead and activated those cells with anti-CD-3, expanded them in IL-2, and then tested their activity.

And since this data was published in JI back in September, I thought I'd just summarize it by showing you here that when we take the D-5 tumor

vaccine and look at the L-selectin low cells, after expansion with anti-CD-3 and IL-2, they are exquisitely specific for the tumor that primed them in vivo, but they're not therapeutic. They're exquisitely specific in that they make Type 2 cytokines. They make IL-4 and IL-10.

When you take our therapeutic vaccine and look at the L-selectin low cells there, you find that they make interferon gamma without making IL-4 or IL-10 and are specific and therapeutic.

So why hasn't someone already made this observation that you see in these poorly immunogenic, in this one poorly immunogenic tumor model, these prime Type 2 cells?

And I think the reason was you need to enrich for these sensitized cells to be able to pick it up above the background, and so thinking about it a little bit more, Hung Ming Hu, who's a Ph.D. student in the lab, knew all of the data that's been out there in the infectious disease literature and the rest of the immunology literature that cytokines derive uncommitted T cells to differentiate along different paths, and if you use anti-IL-12 and anti-interferon gamma and supply a source of Type 2

cytokine, IL-4, you can derive uncommitted T cells to a Type 2 profile.

And you can do that in reverse and use anti-IL-4 and a source of IL-12 or interferon gamma to derive Type 1 cells, and that's been well documented.

So we developed a hypothesis that it would be possible to shift the tumor specific Type 2 response towards a Type 1 response that would mediate tumor regression, and so the experiment was set up like this.

Again, we took out the L-selectin low cells from a D-5 vaccine, and we either cultured them with anti-CD-3 and IL-2, as I showed you before, or with anti-IL-4, IL-12, and IL-2.

And then we looked at their activity both in vitro and in vivo. I'll first show you the in vitro data.

What we're looking at here is interferon gamma or IL-4 secretion. We're looking in picagrams per mL, and these are T cell stimulator with either nothing, with specific tumor, with an unrelated prostate cancer, which is on a black 6 background or 2C11.

And what you see is the black bars of the D-5 vaccine draining lymph node cells, and you can see they've got low levels of interferon gamma and make IL-4. If you look at the ones that were cultured in red with anti-IL-4 and IL-12, you can see they're now polarized more towards the Type 1 and that they've got more interferon gamma secretion, less IL-4 secretion, and they're getting more comparable to our therapeutic vaccine, the D-5K-D, which has got good levels of interferon gamma and low levels of IL-4.

When you ask if these are therapeutic, what you find is that the L-selectin low cells from the D-5 vaccine don't mediate significant therapeutic effects, but if you culture them with anti-IL-4 and IL-12, you see this effect become more prominent, and now it's highly significant, and we've reproduced this in another model, the BALB C-4 T-1 model for breast cancer.

So in summary, what we've seen here is that if you take the D-5 vaccine, which is nontherapeutic normally, and if you take the L-selectin low cells and culture them with anti-CD-3 and IL-2, that you get a tumor specific Type 2 response that's nontherapeutic, but if you culture

those same cells that were primed in vivo with an ineffective vaccine with anti-IL-4 and IL-12, you now get a Type 1 immune response which is therapeutic.

And so what this suggests is that the nonimmunogenic/immunogenic tumor D-5 does, in fact, sensitize T cells to tumor rejection antigens, and that to see those therapeutic T cells, you have to polarize the T cells to a Type 1 response, and then that will uncover that therapeutic efficacy.

So what I suggest is that maybe we've heard a lot about the two signal model, and I think that this is starting to think that there's more than just two signals to this hypothesis, and so while there's certainly antigenic and co-stimulatory signals, there may also be polarizing signals that will direct cells to be one way or another that are probably important in vaccine strategies.

And so the question came up, of course, and you've shown this in D-5: is it only the D-5 tumor model where a correlation exists between this ability where a Type 1 immune response is therapeutic and a Type 2 response is nontherapeutic?

So we've done a number of different tumor models. These first three, the MCA-300 series

are tumors that were developed in my lab by John Osterholtzer initially and Eric Huntzicker characterized them, and the three that I've looked at here are of various immunogenicities. In fact, the MC-304 is very strongly immunogenic. About 90 percent of the animals would be protected by a single vaccination.

While 310 falls more into the weakly immunogenic tumor in that immunization only protects about 25 percent of the animals. There's another series of tumors we'll come back to in a minute.

But when you look at the tumor vaccine draining lymph nodes from the MCA-300 tumor series, so if you vaccinate the animals, remove the lymph nodes eight to ten days later, separate them into L-selectin low populations, and then stimulate them with anti-CD-3 for two days, expand them on IL-2, and then you look at day five for the tumor specific stimulation to see what cytokine profile they make, what you see is an interesting correlation between immunogenicity and the level of Type 1 cytokines that they make.

And the open bars are looking at interferon gamma, and in the solid bars we're looking at IL-4. This is on a log scale.

So the MC-304 tumor, which is strongly immunogenic, has a dominant interferon gamma response with very low levels of IL-4, while the more weakly immunogenic MCA-310 has a strong IL-4 response and a lower interferon gamma response.

So we're looking for a way to somehow look at lots of data and combine it, and this is the mean of three separate independent experiments, and so we're looking for ways to try to look at correlations between whether you had an interferon gamma response or an IL-4 response and how that might work in situ.

So what we did was look at a relationship of a ratio. So if you take on a picagram per picagram basis and take interferon gamma and compare it to IL-4, you can develop a ratio that looks something like this.

And so we've got an interferon gamma/IL-4 ratio here, which is again on a log scale, for six different tumors, and I've got their levels of immunogenicity here on the bottom.

This is three separate experiments in each area. The black bar is the mean of those three experiments, and so if you look at the more strongly immunogenic tumors where 90 percent of the animals

are protected from a tumor challenge, you can see that they've got a very high level of interferon gamma to IL-4, and the ratio is somewhere near 100.

And as you look at tumors that are more weakly or poorly immunogenic where you don't see protection, you see that their IL-4 to interferon gamma ratio, there's more IL-4 than interferon gamma, and in fact, this is probably muted because you're limited by the lower level detection of your interferon gamma.

And so, in fact, we're limiting here, I think, at 20 picagrams per mL, which is our lowest level of detection, and so if you've got 40 or 80 or 80 or 100 picagrams of IL-4 and you divide it by 20, it could really only be one. So they actually may be much, much, much lower.

So in summary, from those studies we've now concluded that development of a tumor specific Type 1 cytokine response correlates with the development of protective anti-tumor response to a P value of .01 at least for these tumor models, and I'd like to say that even though you develop this Type 1 response, those animals, if they were given the vaccine, which is a live, progressively growing vaccine, they will not reject that vaccine. It's

only in a vaccine challenged system that you'll uncover the beneficial effect of developing this Type 1 response.

So our conclusion, at least again in these six and actually now ten different tumor models that we've looked at, that the failure of tumor vaccination is due to the nature of the elicited immune response and not to its absence.

So we thought about this a bit and developed this central hypothesis that the development of a tumor specific Type 1 cytokine response is critical to the generation of therapeutic T cells.

And we thought about some other observations, and the observations were vaccine strategies in naive mice are generally effective. However, clinical vaccine trials generally fail, and we knew that D-5 induces a tumor specific Type 2 response, and thinking about the literature and thinking of immune deviation and functional silencing going towards energy, wondering whether or not does D-5 tumor induce tolerance in the tumor bearing mice and will systemic tumor burden affect a generation of therapeutic T cells from TBDLNR mice

and in the patients that we treated in the vaccine trials?

And what we did is we vaccinated either naive or tumor bearing mice with our aloe modified D-5K-D vaccine and adoptively transferred those activated T cells to other mice that bore tumors.

These are four different experiments. If you just look at the first experiment, what you see is that the D-5K-D sensitizes T cells in naive animals to mediate a therapeutic antitumor effect, but in the animals that were given systemic tumor five to seven days prior to vaccination, they lost all the antitumor efficacy in all four of the experiments I present here.

So we were unable to generate therapeutic effector T cells from the tumor vaccine draining lymph nodes of the tumor bearing mice using the D-5K-D vaccine, and we had the question: how can we overcome this tolerance?

And thinking about the work that was done with the GM-CSF vaccines, which Drew reviewed yesterday, we thought we'd try the GM-CSF transduced D-5 tumor, D-5G6, which was developed in Fred Chang's lab at the University of Michigan.

And what we found there is D-5G6, when you vaccinate naive mice with that vaccine, you get very highly therapeutic cells which essentially cure 100 percent of the mice, and these mice go on to be immune and protected from a subsequent tumor challenge.

But if you do this vaccine in a tumor bearing model, you get a very similar effect. It may not be quite as strong because we have some animals that go on and will die of their tumor, and in this case we're looking at pulmonary mets. So it didn't clear them all out, but generally they're very highly effective.

And just to show you that, and I put this slide in because the discussion this morning when Jeff Sosman raised the question about tumor vaccines and what do they sensitize T cells against, and in collaboration with Elizabeth Tsung initially and Tim Fong and Marty Gidwin and others, and Dale Endoe initially at Chiron Viagene or Viagene and Chiron, we looked at 20 different GP-100 peptides, TRP-2 and some others.

And what we show here is looking at fresh vaccine draining lymph node cells just pulsed with peptides and put into ELISPOT assays, and the

other peptides are essentially absolutely negative, but what you can find is ratios or spot forming cells, frequencies of interferon gamma secreting cells to at least these five different GP-100 peptides that range somewhere between one in 6,000 and one in 8,000.

And then if you take those cells, expand them with anti-CD-3 and IL-2 and come back and pulse them with peptides, you can see specific cytokine response to those peptides.

You can also expand those cells up and adoptively transfer them, and they mediate antitumor effects.

If you then look at though, getting back to the tumor bearing mice, so I told you that we took D-5K-D vaccine, and we tried to take those and we put that into a mouse that has a systemic tumor burden, and we were unable to generate therapeutic cells from there.

So what was the phenotype of those effector cells that we were transferring into the mice? And when you look at their cytokine profiles, and I'm comparing here the naive mice in the open bars and the tumor bearing mice or the effector cells from the tumor bearing mice in the red bars,

you can see that they've got a decrease in tumor specific interferon gamma, but they still have some tumor specific interferon gamma, but these cells are completely ineffective.

If you look in the D-5G6 vaccine in the tumor bearing mice, you can also see they've got a big reduction in tumor specific interferon gamma, but yet' they're still highly therapeutic and will cure animals and provide long-term immunity.

So there are so many questions there. It's not what we thought it was going to initially be with immune deviation where you'd polarize the Type 2 response because essentially the IL-4 data, which I didn't show you, is negative, but there's some other mechanism, and maybe it's functional silencing going towards energy that's happening, and that's a current effort in my laboratory that we're working on.

So a summary from those studies is that the GM-CSF modified tumor vaccine can break this what we're thinking of as tolerance in loose terms in the tumor bearing mice, and that these therapeutic T cells have reduced tumor specific Type 1 cytokines.

So we also have ongoing a series of clinical trials based on our animal work, which are being done in collaboration with Walter Urba, my clinical colleague, and John Smith and several surgeons back in Portland, where patients are vaccinated with their autologous tumor that's either been aloe modified to express HLA-B7 or mixed with BCG, and the tumor vaccine draining lymph nodes are removed seven to ten days later. They're activated, expanded, and given back.

We've had extra cells on a number of those patients, and so we've separated them into L-selectin low and L-selectin high populations, expanded them with CD-3 and IL-2 to see what their cytokine profile was and whether or not we could actually enrich for the tumor specific T cells in these patients.

And what I'd like to say first is if we take the total population that we're able to generate tumor specific cells, tumor specific cells in fact that in the case of both renal and melanoma, that will recognize certain other aloe melanomas, but not a whole panel of other melanomas that are aloe, and we're currently in the process of looking

at whether or not in the melanoma case which GP-100 peptides they recognize.

But what we've seen is when we look at the bulk population that we activate with anti-CD-3 and IL-2 and these are the cells we give back to the patients, we see a mix of both Type 1 and Type 2 cytokine responses that appear to be specific in many cases for the patient's autologous tumor, and that when we look at the L-selectin low population, they seem to be enriched more for Type 1 rather than Type 2 cytokine profiles.

And interestingly enough, we thought that the L-selectin high population, which should be naive cells, would actually have a lower frequency like we saw in the mouse of tumor specific cells, but, in fact, we find that they've got an enriched population at least in one of the patients that we studied so far, and I would say that these are so preliminary studies that we've only looked at four patients, but we've seen that at least in this one case, we saw an enriched Type 2 response in the L-selectin high population.

Then we went back to the literature and found a report from a Japanese group that said that, in fact, that was what they had found as well.

But more importantly, because we believe that in our mouse model and we want to translate the mouse model to the patients, we looked at whether or not we could take the TBDLN that we have, that we knew were giving us mixed Type 1 and Type 2 responses, and whether or not we could polarize them with an IL-4 antagonist and expand them in IL-2 and develop a Type 2 response.

And these were studies that were done in collaboration with Raj Puri, who provided the IL-4 mutant protein.

And what we've shown is that the mutant IL-4 can inhibit the development of T cells with tumor specific IL-10 secretion, which we're taking as a Type 2 cytokine profile, and what we're looking at here is we're looking at tumor specific looking at IL-10 or TNF beta secretion, TNF beta being another Type 1 cytokine, and we're looking at T cells cultured alone without any other mutant IL-4 or with an escalating dose of mutant IL-4 added during the culture period of 210 or 100 picagrams per mL.

I can't see the colors very well, but I think the black bars are the T cells alone. The red

are the bars of the T cells cultured with autologous tumor or an unrelated renal cell 79 tumor.

And what you see is you see IL-10 secretion that goes down with increasing, escalated dose of mutant IL-4 protein, while you maintain your Type 1 or TNF beta secretion with those same cells.

And so our hope is that this would be a source of Type 1 cells that we could use in adoptive transfer studies.

So tumor progression in a Compton host may be explained by a number of mechanisms, and I'm not trying to say that immune deviation is the answer, but certainly T cell ignorance has been well documented, and energy is also a possibility for why tumors progress.

Immunosuppression with different molecules deletion, but I just want to say that immune deviation is another alternative, and I'll stop here, in my concluding.

There's a lot of people in my lab many of whom have moved on, but John Osterholzer and Eric Huntzicker helped develop the 300 tumors. Hong-Ming Hu has done the majority of experiments that we've presented. Hauke Winter, who's a surgical resident,

and Joffer Bashi and David Lashley who are also urology residents in the lab did the prostate stuff.

My clinical colleague, Walter Urba, who allows all of this to happen and helps us translate it into the clinic.

John Smith, Bill Wood, Bruce Lowe, John Bettle, surgeons that have worked with us. Andy Weinberg, Pete Boyd Cantab who have helped supply things. Raj, people at Viagene who have helped us with the GP-100 peptide stuff, and people at VICAL, Mick Croft and Carl Ware and Bob Mitler with some 4-BB stuff I didn't get a chance to present.

Thank you for your kind attention.

(Applause.)

DR. SZNOL: Would all the members of the discussion panel please come up, and, Pat, I believe you're chairing this session.

DR. KEEGAN: All right. Well, the issues to be addressed during this panel session would be some considerations about these immunological assessments that might help us guide in the clinical development of these agents.

And some of the concerns that we have in terms of the immunologic assays are, first, that they would be relevant to the effective mechanisms

for tumor vaccines; that they can be standardized and reproducible assays, which are important both for the initial development, but even more importantly for advanced development in Phase III trials where considerations of reproducibility across centers or whether or not there needs to be a centralized laboratory in place.

That there be some evidence of discriminatory ability, which again is important in the initial studies for dose and schedule optimization, but also for considerations of post marketing bridging type studies when there might be modifications of the product, that one might be able to use some of these assays for such bridging studies as one does in prophylactic vaccines for infectious diseases, as well as some issues regarding characterization of patients' immune states in a Basil prevaccination and how those issues might impact upon assessment of the immunological assays.

So I guess the first question really addresses the one about the relevance to the effector mechanisms and how does one go about determining which assay to utilize, what kinds of in vitro and preclinical animal models are useful in

choosing the appropriate assay to assess immune responses in the clinical trials, and what kinds of things would one want to control for, assess in the patient population to determine whether or not these have impact on the immune responses.

And we could probably just start if anybody wants to start with the initial question or we could go down the panel, and now from Jay's side.

DR. ROSENBERG: You know, with respect to the assays used, I think we heard a lot about the many different assays today, but we need to remember that virtually all the assays that we discussed were measurements on peripheral blood, and peripheral blood may be exactly the wrong place to be looking.

We can measure with the peptide vaccines very high precursor frequency levels in the one to two to 3,000 range in many patients. Again, that's what you would get after infection with flu, and yet in the face of those high precursor levels, patients do not show responses.

And, again, so long as the cell is in the circulation, it's not likely to be able to impact on a tumor. It has to become extravascular and get into the tumor stroma. So we need to be

emphasizing somewhat more looking in the tumor stroma.

Further, when we give Interleukin-2 along with these peptide vaccines, even now, even though 40 percent or 42 percent of the 31 patients in our trial responded, the measurement of precursors in peripheral blood did not increase. It decreased significantly, and we don't have a good explanation for that, but perhaps it's because those cells left the circulation, indirect tumor sites or go to tumor, and in the face of IL-2 or apoptosed, we don't fully understand that.

With respect to the relative sensitivity of the assays, we have in those peptide vaccine trials directly thus far compared the bulk cytokine release assays, ELISPOT assays, limiting dilution assays, and now with Drs. Lee and Mirankle the tetramer assay, and nothing in our hands has been more sensitive than just a bulk assay of peripheral blood mononuclear cells that are exposed to a specific peptide.

The tetramer assays, which are elegant, are, of course, limited by the sensitivity of FACS, and so one can't reproducibly see precursor frequencies that are going to be less than one in

1,000 just because of limitations of the background noise in most FACS assays.

DR. KEEGAN: Dr. Berzofsky, would you like to comment?

DR. BERZOFSKY: I think I agree with the comments that Steve made about looking in the peripheral blood unfortunately may not be the optimal site to look even though that's what we have access to.

We haven't done the comparison that he just described between these different assays. We're trying to set some of them up, but in infectious disease models, we've certainly been able to see a correlation between CCL lytic activity and virus clearance, as well as between interferon gamma production and virus clearance.

And I don't know how much that will correlate, of course, with tumor clearance, but certainly in those animal models you can actually show cause and effect in some systems.

I think it remains to be seen exactly which are the right cells we should be looking for, and we've heard today examples of cytotoxic T cells, as well as examples where TH-1 responses compared to TH-2 responses were very important, and ultimately I

think all of these, as well as potentially antibodies, may play a role.

So I'm not sure we can limit ourselves to any single assay at this point because each of those will give us a different window on the response, and until we have real clinical correlates, it's hard to know which of the responses will best correlate with clinical responses.

DR. SCHLOM: I'd like to ask the people working in the clinical melanoma setting have they looked at different time points at precursors, one week after immunization, two weeks. Most people are taking their samples, I think, three or four weeks after immunization. Has anybody looked at temporal factors?

DR. FOX: Actually we have. We have, too. I think Fred Chang has got data on that as well, and that is that when you vaccinate and look at one week after vaccination, and we have the draining lymph nodes to go in parallel with that, you can find high numbers of tumor specific cells in the draining lymph nodes while the peripheral blood are uniformly negative for the same cells.

So activated with CD-3 and expanded in IL-2, they have no antitumor activity, whereas you

can find in some cases a high frequency of tumor specific T cells in the draining lymph nodes.

PARTICIPANT: Maybe I can comment on this because we've done serial kinetic studies of peptide specific CD-8 responses following multiple immunizations, and measuring CD-8 responses following two, four, and seven immunizations, what we found is that the peak of the response was after the fourth immunization, and after that the response fell off.

What isn't clear is whether the response fell off at that point because the immunization schedule became longer, that is, it was a longer time, you know, between immunization.

But clearly the kinetics of the response are very important, and the time you pick to measure your assay is going to impact on the results that you get.

But, you know, I'd like to make one comment if I can, which is that I'm not sure that at the present time we really know, you know, what the most important parameter of the immune response is that we ought to measure to look at protective effect.

I mean, clearly CD-8 responses are very important. There's considerable evidence that antibody responses are important. There's evidence presented by Dr. Morton, and we've had, you know, similar data that the combination of responses correlates best with improved clinical outcome.

Other cell types would be important, CD-4 cells. NK cells may be important, particularly in advanced tumors when the tumor may lose HLA molecule and, therefore, will no longer be susceptible to be killed by CD-8 cells.

So one point which I would like to stress is that at least in the scientific evaluation of vaccines, not necessarily in the regulatory aspect, that one measures multiple parameters of the immune response until we have a better sense as to what is really important, and most likely what will turn out to be important is that all of them will be important to different degree and in different stages of tumor evolution.

PARTICIPANT: Could I just extend that comment? I also feel badly that antibody has gotten such short shrift.

(Laughter.)

PARTICIPANT: When you have minimal residual disease where antibody has access to the tumor cells, we already have several instances clinically where they have been very effective.

But the problem is that people expect them to be effective because of their conventional effector mechanism, bringing in ADCC, fixing complement, et cetera, but in fact, antibodies properly used which multimorize (phonetic) the receptors are very powerful as agonists in inducing apoptosis and cell cycle arrest.

That's how they should first be screened. Then you could put on the right cassette to get a maximum effector function, and I think in that setting, where you have access to cells, where they have powerful signaling effects and effector effects, they will synergize with T cells, and that's what we've observed in a mouse model where we can induce dormancy by a very strong anti-id signaling antibody, but T cells synergize with the antibody.

DR. KEEGAN: Okay, yes.

DR. WEBER: One piece of mouse work that's recent suggests that during and just after the peak of a viral infection in a mouse, the

convalescent levels of CD-8 positive T cells are extremely high. It's not one in 5,000, not one in 1,000. We're talking ten, 15 percent of all the CD-8 T cells may be viral specific.

And it could be that one in 5,000 or one in 2,000 is simply not a threshold level of CTL that are antigen specific in melanoma to induce either protection or aggression. It could be that you literally have to get up to one in ten to be able to see a positive clinical effect.

I'm not saying that's the case. I'm just throwing that out as a thought, that we may be way off in the potency of our immunizations. If we can generate one in 1,000, for example, it may just be off by a factor of ten or more.

DR. KEEGAN: Dr. Lee, could I ask you to comment on the tetramer assays and whether or not -- most of the assays that we've heard about are directed at some functional assessment, and I think the tetramers are in contrast to that.

Do you think that the tetramer assay should always be evaluated in conjunction with some functional assay or in conjunction with assessment of CD-45 molecules?

DR. LEE: Yeah, I think one of the things that I really like about the tetramer approach is that you can isolate these cells with a minimal amount of perturbations to the cells. You don't have to stimulate them in vitro. You don't have to culture them with cytokines.

And so it's about as close to the native state as you can get, and so I think because of that it's a fairly powerful method of studying the native biological properties of these cells, such as surface markers, et cetera.

DR. KEEGAN: I guess what I'm working toward is how would one go about looking at how it is that whatever it is you're measuring relates potentially to the desired effect in terms of tumor reduction, and it sounds like most people are suggesting that we don't know so that we need to use multiple assays.

But to what extent, because that may be somewhat impractical to do in clinical trials, to study everything simultaneously -- how useful are the animal models in terms of selecting or minimizing the number of assays being utilized?

DR. KHLIEF: Actually, if I may add on this question, when you did the tetramer assay for

Jeff's trial, did it correlate at all or what was the correlation with the immunological assays that they conducted, like cytotoxic assays or cytokine releasing?

DR. LEE: Yeah, we haven't really gotten together to really sort all the data out yet, but right now it looks confusing.

(Laughter.)

DR. WEBER: Samir, the one patient that Peter had the best response in did have immunologic response, but I would call it a modest immunologic response. There was obviously more cytokine release post and pre, but it was not as impressive as the relative numbers, the ratios of cells, the CD-8 cells that were positive post/pre in the tetramer assay.

I mean, you know, you need to get enough numbers. You need to get 20, 30, 40 patients to make some comparisons, and that's what we're going to try to do.

DR. KHLIEF: You know, I think I would add just one point that I'd probably stress at this stage on immunological monitoring mainly, and this is not a statement. Probably it's old for discussion. Somebody yesterday said just do

whatever you believe in at this stage from an immunologic monitoring point of view until you reach to a point where you have an antigen that lead to a good clinical response that you can correlate with what you have immunologically and say this correlates and this doesn't correlate. That might be the approach at this stage.

DR. KEEGAN: So are you saying that it's not until the Phase III trial is completed that one can go back and try and elucidate what's an appropriate immunologic response which might subsequently correlate?

Dr. Simon, I can see you.

DR. SIMON: If you had the data to know what was the best immunologic assay, you really probably wouldn't need the immunologic assay at all because you would be having enough clinical responses to be using clinical response as your endpoint.

So I think by necessity since you're dealing at a level of study in which you're not getting a lot of responses, you basically are having to use your best science and are essentially fishing around based on your best, you know, animal models or whatever to use the assays you think are the most

relevant, but you may be wrong and they cannot be validated.

DR. KHLIEF: Actually this is why I said it's not a statement. It's a discussion, but you know, I might disagree on this because if you have an indication from one antigen, for example, that this particular assay could correlate, that assay might be used to know whether that particular antigen has potential in the future. If it was a weak, then you would grow an enhanced on that antigen to reach to the clinical responses if that's the case.

PARTICIPANT: Or better explain nonresponders.

DR. KHLIEF: Or better explain nonresponders, absolutely.

DR. KEEGAN: Because we're going to be moving from Phase I into larger trials, it's clear that one needs to be using assays that are going to be reproducible at different centers. To what extent do the assays that are currently in use -- are they the types of assays that could be performed reproducibly at different centers in such a way that one could utilize the data or pool the data?

DR. DISIS: Some of the assays are very easy to do and quite reproducible, and some of the assays are very difficult to do and require special techniques, and that's why I think that there are several cooperative groups around the United States that are looking to run Phase II studies of promising vaccines that have been tested in Phase I studies, actually sending samples to centers that have the expertise and some of these more technically difficult assays.

Because I think it's the feeling of most people that there are several good strategies out there right now. There are several good antigens that would allow monitoring of an immune response, and that what really needs to be done at this point with some of the very quantitative assays that require very little or none in vitro manipulation, that we really need to show that the immune response correlates to a clinical response, and that can only be done in terms of Phase II study.

DR. KEEGAN: Dr. Berzofsky.

DR. BERZOFSKY: I'd like to mention one result that adds support to the use of cytokine measurements as a key response that Bernie Fox mentioned and that Steve mentioned as well, and that

is an epidemiologic cross-sectional study that was done by Tagret Sekui in our lab in collaboration with Alan Hildesheim and Mark Schiffman's group, looking at women with different stages of cervical neoplasia related to papilloma virus.

And we looked at the IL-2 response to human papilloma virus antigens, and there was an inverse correlation between that and the degree of cervical neoplasia, and it was antigen specific.

So that's a human epidemiologic study that suggests a correlation between a TH-1 type response and lack of progression, although it's done in a cross-sectional, not a longitudinal study. So you can't prove cause and effect, but it certainly supports that kind of interpretation and fits nicely with the data that Bernie presented earlier this afternoon.

DR. KEEGAN: Dr. Siegel.

DR. SIEGEL: It struck me in listening to the panel that if this were five or ten years ago, we would have heard perhaps a lot more about CTL assays using chromium release in tumor killing. Jay, I think you had some data on that, right, with lytic units, but not a lot, and I wonder. Is there a consensus? We're hearing a lot more about the

cytokine assays, some about proliferation, two cell enumeration, flow cytometric assays. Does that reflect the difficulties of doing the CTL assays in a reproducible cross-sectional way or a feeling that maybe they're not giving the more important answers in terms of optimizing vaccine strategies?

DR. ROSENBERG: You know, I don't think it really makes sense to be thinking in terms of what immunologic assays we should be using to monitor vaccine trials because unless you are performing a manipulation that causes a significant number of clinical responses, you cannot correlate what in vitro assays are correlating with clinical responses.

And so we perform the immunologic assays to try to understand the immunologic impact of what we're doing, but to talk about using them as a monitoring tool really makes no sense because we can't perform the correlations unless clinical responses are seen.

In these two days we've heard very little in the way of clinical responses to virtually any of the vaccine manipulations that have been performed in humans.

So I don't know how we can talk about what is the most relevant assay. We can only talk about how can we study the impact of the manipulation.

DR. FOX: Just an additional comment on Jay's question. I think though, too, that in our cases, the T cells that we're adoptively transferring back are not cytolytic, and so we've stopped doing it because we don't see much cytolytic activity.

At the same time, we see strong cytokine releasing responses from those T cells. We also have data in the mouse that shows that in this Type 1/Type 2 paradigm, you can do these experiments in perform knockout animals and see perfectly good tumor regression and development of immunity.

So we think of it as being certainly a cytokine based mechanism in the absence of perform that can cause complete tumor regression and immunity. So we've kind of gotten away from that, too.

DR. WEBER: I have two quick comments. One is certainly to agree with Steve that you have to be, as we discussed yesterday, you have to be able to correlate what you're studying

immunologically with some clinical beneficial effect. It could be clinical response. It could be time to relapse. It could be overall survival. Any of those, I think, are reasonable surrogates for clinical benefit.

The other is if I had to choose an immunologic monitoring test, I would think about something that would have some sort of internal control, and flow cytometry using the tetramers, just as an example, or just flow cytometry in general, to agree with what Gerry Marti said and what Carleton Stewart said, would seem to me to be the most reproducible and promising type of assay.

I don't mean tetramers specifically, but a flow cytometrically related assay since there is significant uniformity available among the machines. You can have internal controls and set them.

I would look to some flow cytometry assay in the future, but, again, it doesn't matter what kind of assay you have. If it doesn't correlate with a clinical beneficial effect, who cares?

DR. SIEGEL: Well, still, I think the point needs to be repeated. I think we're all in agreement there are, as was well pointed out, there

aren't clinical data. No one's going to correlate an immune response with clinical data, and it's easy; it's possible to say, and it's correct to say that one should measure all of those immune responses so that when you get clinical or as many as you can reliably, so that when you get clinical data you can correlate it.

But, in fact, those trials to get clinical data are going to take years and millions of dollars. There's only going to be a limited number of regimens that go forward. We saw the panoply of choices that need to be made.

What is the dose? What is the regimen? What are the adjuvants? How are we going to do that?

And I think virtually everyone we heard from is using some immunological marker, some immunological marker to optimize or select among those regimens, and ultimately how well we guess what's the right one is a very critical question as to whether those agents that go forward into clinical trials have a good likelihood of success.

DR. KEEGAN: We can take a few questions from the audience, I think.

DR. MARTI: I have a question for Dr. Lee. First a comment.

A historical thing on the CD-34 determinations, which on a good day might be as high as some of your antigen specific T cells. The interlaboratory variation at least in North America has been recorded as high as plus or minus 1,000 percent.

I won't ask what your positive control is for enumerating that low a percentage, but I had another question that I wanted to ask you.

Oh, does each patient have to have his own HLA type Class II antigen made or is it generic?

DR. LEE: Class II?

DR. MARTI: Well, when you make this tetramer, like if you're going to immunize me, do you have to know my HLA type, and if we're going to immunize you, do we have to know your HLA type?

DR. LEE: Yeah, exactly. So you have to know both the HLA type and the peptide to make the tetramer.

DR. MARTI: So this is like a customized hybrid owner.

DR. LEE: That's right, and so for practical purposes we've stuck to HLA-2.1.

DR. MARTI: Okay. So you choose one that you can screen and study a lot of people.

DR. LEE: Yeah, but your point is well taken. For FACS analysis, the problem is that your numbers will change depending on the gaits that you set, and so it's a subjective thing. Unless you get a very, very clearly positive and very clearly negative population, it's always going to be this kind of border that will affect your numbers depending on how you set the boundaries.

DR. MARTI: But it can probably be standardized and controlled. I would approach it that it could be because it certainly has been going in that direction for CD-34.

I was hoping that you'd recommend that all of the investigators in this room all need to have a nine color flow cytometer.

(Laughter.)

DR. WEBER: I mean, because the peptide and the MHC are going to match up, and with five haplotypes you can cover the whole population. So I don't think it's a valid comparison, although you will have to match the peptide with the particular ileal, and you'll have to haplotype the patient, but that's not that difficult. That's standard stuff.

DR. MARTI: So those reagents will become available within a month.

DR. WEBER: Actually some of them are already commercially available, by the way.

DR. RESTIFO: You know, for all of the kind of feeling that we don't know what to measure, I'm a little more optimistic than that. I think that, I mean, we do know a little more than that. We know antigens that are expressed on the surface of tumor cells that are restricted by MHC Class I molecules. We know their identities.

Now, not every antigen is going to be expressed in the surface of these, but we know some. We know that cytotoxic T cells are found in tumor beds, that they can recognize human tumors ex vivo. We know in animal models that pure populations of CD-8 positive T lymphocytes can be transferred and can recognize tumor cells. That's why we measure CD-8 positive T cells.

That doesn't mean it's all CD-8 positive T cells. We also know that COGME CD-4 help in some models, can alter or can help CD-8 positive T cells by secreting IL-2 and other co-factors. We know that CD-4 positive T cells can alter antigen

presenting cell function, can super activate dendritic cells and other antigen presenting cells.

And so I don't think we need to be so bleak about our state of knowledge. I think it's a heck of a lot better than it was ten years ago or even five years or three years ago, with all of the molecular characterization that's gone on.

So I think that moving towards a state of reductionistic analysis of what's going on on the molecular level is what's going to get us places.

DR. KEEGAN: Dr. Simon, would you want to give a final word, a little bit about any strategies for looking among different monitoring -- among the different assays and using or selecting those from the results of trials, since it seems to be that people are suggesting we're going to be using the clinical responses to drive selection of some of these.

DR. SIMON: Well, I agree with much of what has been said about the general overall strategy, including the way Jay put it, that we have to optimize various aspects of the delivery of the vaccines and we don't really know. We have to use our best science, and we have to make some judgments as to what types of immunologic endpoints to use.

I guess the one thing I feel that we really need to make sure we place attention on, in addition to the science of the immunology behind the assays we use, is a lot of attention on the reproducibility of the assays we use because I think if we don't really make sure that whenever we're going to do a clinical trial that we really understand in context of that trial for that peptide or that antigen what the reproducibility of the assays we decide to use are, then I think we're really limiting ourselves because I think then the interpretation of individual trials -- and I don't mean just reproducibility if you take one sample and put it in 24 wells. I'm talking about reproducibility either in multiple blood drawings or if it's a trial that involves multiple laboratories, then it has to include that component of reproducibility.

That really, I think, requires professional attention to make sure that whatever assays we use, that the results are interpretable.

DR. KEEGAN: Okay. I think we -- okay. One last question, and then we'll go to the break, I guess.

PARTICIPANT: Well, maybe I can make two brief comments and two brief questions.

(Laughter.)

PARTICIPANT: No, the comment is that -- the comments are that I think we should be much more positive about the use of immune assays to monitor the effective of vaccine treatment. I mean, these assays are available, and the resulting assay is really what we must use in Phase II trials to optimize the way we administer and make the vaccines.

I think that there are ways of correlating the impact of the vaccine to clinical outcome because the clinical outcome points that you have are not only whether or not the tumor goes away, but you can use endpoints like disease free survival, overall survival, and you can make correlations, and these have been done by a number of investigators.

One thing that I think you must be conscious when you make these correlations is that association doesn't necessarily mean causation, and there may be different factors that account for why a patient does better or worse.

I think just like we do when, you know, you evaluate the impact of a drug treatment, its standard approach to do multivariate analysis. You take into account the risk factors that can impact on outcome.

The same way when we look at the impact or associate the result of an immune assay, its critical outcome, it's very important that we do Cox multivariate type of analysis to try to control for other factors that could impact on outcome.

DR. KEEGAN: Okay. I'd like to thank the panel and say that we're going to take a slightly briefer coffee break, about ten minutes.

(Whereupon, the foregoing matter went off the record at 3:40 p.m. and went back on the record at 3:57 p.m.)

DR. GREENBLATT: So I would like to get started. So those of you who are in the room, if you could take your seats, we can start.

My name is Jay Greenblatt. I'm from the Regulatory Affairs Branch, Cancer Therapy Evaluation Program at the National Cancer Institute, and one of the organizing committee of this meeting and co-moderator of this last, but not leastly important section on detection and characterization of tumor

antigens and vaccines, and which also has relevance to other fields, such as bone marrow transplantation of cancer patients.

It is with great pleasure that I get to introduce my co-moderator of this last session and someone whose name you are all familiar with, Dr. Jonathan uhr, now retired Professor of Microbiology at University of Texas, Southwestern Medical Center in Dallas, and his presentation is entitled "Detection and Characterization of Carcinoma Cells in the Blood."

Dr. Uhr.

DR. UHR: Can you all hear me?

PARTICIPANTS: Yes.

DR. UHR: It's an intimate group. So I won't have to speak quite as loud.

(Laughter.)

DR. UHR: Of course, I want to thank the organizers. I've learned a lot from this meeting.

I'm going to describe to you a very sensitive test for enumerating and characterizing carcinoma cells in the blood. Its pertinence to the theme of this meeting is obvious, but I do want to discuss with you first the idea that made me develop

the test, and at the end what I'd like to use it for.

Now, the hallmark of successful treatment is earlier detection. Can I have the first slide, please?

And basically this is really the hypothesis, that when you have a small number of tumor cells, perhaps ten to fourth to the sixth, they're already shedding. They break down tissue barriers. I don't see how they cannot be shedding into the blood, but I think cells at this point for the most part become apoptotic or dormant.

Now, as the tumor grows and, of course, is genetically unstable and more genetic changes take place, you reach a point where you can detect it by sensitive conventional assays. Let's say mammography can detect may be as little as two times ten to the eighth tumor cells, not less.

And at this point in time, I still think you have most of the cells or markers for the tumor, but will not metastasize, but in some cases, yes, we know that even a small breast tumor, less than a sonometer in diameter can have metastases, and with angiogenesis have progressive growth.

Now, there are a large number of tumors relatively inaccessible -- the pancreas would be the example par excellence and others -- which are routinely detected very late and very often have local invasion and growing metastases.

So the hypothesis is that tumor cells should be present in the blood by Stage II and probably earlier. Now, you may think this is a bizarre speculation that we'll detect it here, but I will raise it for you because it has implications.

If one detects the cells here and one can prove that they're neoplastic cells and you have the organ of origin, not hard to do in the breast and prostate, you might want to vaccinate at that point, and you'll have a very small number of cells that are less genetically screwed up, and they might be more easily handled.

Now, the test is -- this is the objective to develop the test, and the test is a two phase test.

Is this working?

Where we take a small amount of blood, ten or 20 mL. We put on ferrofluid, which is a colloidal iron suspension. These are not particles that you can see. They're submicroscopic, about 150

nanometers, and you coat them with the right anti-epithelial adhesion molecule which has high affinity and some other characteristics we can't really detail.

You can with appropriate washing and magnetic field get a 10,000-fold purification, and you have to get that for the test to be effective.

Then you can use flow cytometry. You can use a dye to not stain the red cells and, therefore, exclude them, anti-CD-45 to exclude the white cells, and then we use an anti-cytokeratin to again pick up the epithelial cells, and one can do others. And, in fact, we're planning to use as many as six or seven because it is possible to do that now.

Now, you could also take after this purification, cytospin the cells, and look at their morphology, their immuno-histochemistry, and analyze them genetically. I mean multi-color FISH on interface cells is a very effective way of doing this, and we're collaborating with Thomas Reed here in those studies.

The cells that come from here are alive, and they stay alive for a while in culture. We haven't tried to establish a cell line from them,

but perhaps in the future they could be looked at as a testing ground for potential therapeutic agents, looking for apoptosis, for example, with membrane flipping.

Now, the first question to be asked in terms of the test is how efficient is it. Can you recover all the cells? And to answer that you simply mix normal blood with different numbers of adenocarcinoma cells, and they can come from any kind. In fact, the ones I show you come from a colon carcinoma, and perform the immunomagnetic purification followed by FACS.

And the answer is that when you put in no cells, you get back none. We've painted the epithelial cells red. You can see this is CD-45 on the abscissa, and cytokeratin on the ordinate.

If you put in 200, we get back about 75 percent, but some of these dots are superimposed. When you get down to lower numbers, you get them all back.

So recovery is at low numbers, which is what we're interested in, is very high, above 95 percent.

Now, the next question was: can we detect these in the blood? And we want to look at

normal individuals. We want to look at patients who have early cancers. If we can't detect them in those patients, then we might as well stop the study.

So again, we'll do the complete analysis on normals, patients with nonneoplastic diseases, patients with clinically organ confined carcinoma, and patients with metastatic carcinoma.

And when we did that, here are representative examples. A is a normal person. He has two epithelial cells. About 40 percent have none. The other 60 percent of, quote, normals can have up to five epithelial cells. We don't know their source. It may come from putting a needle through the skin. It may come from shedding from a mucus membrane, but there are small numbers or none.

Now, here is a patient with breast cancer, organ confined. This was taken just before surgery, and there are nine cells, and here's one that has an organ confined prostate cancer. I think there are ten or 11.

This is a patient with breast cancer, but it has metastasized already. There's a very large tumor burden. I think I calculated this as many millions of cells in the blood.

Now, to summarize, is there a statistical difference between the control groups and the patients with clinically organ confined carcinoma? We're not interested in diagnosing metastatic cancer. That can be done clinically.

And we simply looked at coded samples by FACS. I have to tell you, I mean, FACS I do feel has subjectivity, and that's why I had the samples coded, and in fact, they were done by two different observers. One did the gating individually, and the other used an algorithm.

If you use an algorithm, it will be objective.

And there was no significant difference between the two, and here are the results.

Here are the normals in the white. There's no difference whether they have a benign tumor or not, and they usually go up, as I told you, to five cells. You recall can't see them here because they average, I think, 1.7 cells.

The organ confined; there were 26. Seven were prostate. The rest were breast, and in 24 of the 26, they had seven or more cells. The average was 16 in each, which is purely coincidental.

We calculated a cutoff point based on this 6.8 cells, the standard deviation, the average plus three times the standard deviation. So 24 of the 26 we're able to diagnose by count alone.

I should say a tentative diagnosis. It was highly significant statistically by several statistical methods, and there's a statistical difference between the organ confined clinically and the metastatic, again arguing that we're looking at tumor load.

Now, are these excess epithelial cells carcinoma cells? I mean a dot on the plot is not the same as a carcinoma cell.

So for this we did our immunomagnetic purification, which still gives us a lot of white cells on the slide, but not so many that we have to use more than one small area of the slide.

Of course, we coded the slides. We stained them with Mucin-1, which tends to stain only malignant cells intensely, and we looked at them or I looked at them for cytomorphology, again, of course, coded.

And I'll show you what they looked like, and I'll tell you how well I did in terms of the coding. Here are normal epithelial cells. These

were obtained from foreskin. We didn't want cultured cells because these aren't cultured. They have a lot of cytoplasm compared to the nucleus. The ratio of nucleus to cytoplasm is relatively low. It's a rather eukaryotin type of nucleus and so forth, doesn't stain with Mucin-1, and notice that these cancer cells, these two are from organ confined, I believe, breast and carcinoma; have a huge nucleus, just a rim of cytoplasm. Cytoplasm stains heavily with anti-Mucin-1. The nucleus has rather disorganized chromatin clumps and easily distinguished from the normal cells.

Here is a macrophage with two tumor cells that I think are consistent with apoptotic tumor cells.

Now, when I looked at these slides, I made no false positive calls. There was no interobserver error. I was given some slides two times, but I did miss two prostate carcinomas, which I called normal, and frankly, I think it's a function of my age and patience because the postdoctoral fellow showed me afterwards on at least one of them a clear-cut carcinoma cell, but you know, when you begin to look at two or three dozen slides at my stage, you get a little impatient.

But the key thing was no false positives and no interobserver error. One can afford a false negative for the reasons I mentioned.

Now, the next question was: does the blood test for the epithelial cells parallel the clinical course? Would this be at all useful to monitor therapy if one wanted to?

And 12 patients undergoing treatment for breast cancer were followed clinically blood test for one to ten months.

Now, one thing I'll tell you. A caveat about this experiment is that it encoded. The clinician knew the blood tests, which I'm not happy with because I like to have these things not done that way.

But anyway, in four representative patients, you can see that if you plot the clinical status, which is measured no evident disease and stable disease and progressive disease, life threatening disease, et cetera, versus the number of epithelial cells in the blood, which we now know are carcinoma cells, you can see that there's a general parallelism.

High dose chemotherapy and both fall. There's a relapse rather quickly, and again they're

given high dose chemotherapy. It falls again. On maintenance chemotherapy, lower doses, it falls, but then it comes back up again, et cetera.

Notice this patient that had no evidence of disease basically had relatively small numbers at this point. These others are much larger, as you see, in terms of the ordinate.

So I guess you can consider this another argument that we're measuring tumor burden, and it may be useful basically to monitor treatment.

Now, what is the difference between this blood test and a lot of other assays for tumor cells in the blood that have been used frequently? And why are we able to detect such a small number?

Well, the first thing is the sensitivity. I mean, you can't use large metal beads. You can't have any clumping. You have to get a 10,000-fold purification. The beads we're using do not have to be removed. You can't even see them.

So I think that's one of the key things, and I think the flow cytometry has to be done very carefully and properly, and if, for example, you lysed the red blood cells, the noise level is too high.

Well, the fact is that I think that the volume of blood we take is the limiting factor. I think we can detect one carcinoma cell in 10 mL of blood.

PCR based methods can possibly go up to one in ten to the seventh, but that's not usually the case, and you certainly can do it by magnetic enrichment with immunocytochemistry. Both of these can look at the cytology, which you can't by PCR, and here you can quantify the number of cells quite precisely. You can't do that. It's really positive or negative with PCR. You can't do it with immunocytochemistry.

Now, with both of these you can look at both proteins and nucleic acids. Of course, you can keep on going back to the same cell and look at different markers in each of these methods. You can look at markers for proliferative status, invasiveness, aggressiveness, et cetera. I've presented none of that data to you.

You can compare the primary tumor to the cells in the blood to be able to say that the blood cells come from the tumor cells. It's something we're in the middle of right now, and I can't talk about it yet. We're too early in the game.

One may be able to do this to some extent with PCR, but it's limited, and of course, we can get viable cells. We haven't exploited that option yet, but it might be worthwhile.

Now, in terms of the theme of this study or I should say of this session, I mean, I would think that if we get further with our method that we should be able to be of help to you in terms of accurately determining the number of tumor cells that you have and their quality, and I think the latter is going to be more important.

I mean, our current plans are basically to take 30 mL of blood, use 15 mL for more immunophenotyping and to look at other antigens that will be helpful in terms of determining the organ of origin of the tumor, I mean, mammoglobin for breast, PSA for prostate, et cetera, and use the other half for multi-color FISH.

And you know, changes in copy number of proteins to the chromosome are seen very early in the game in breast cancer, and Dr. Reed with just a small number of probes has been able to detect these, and of course, there's an unlimited number of probes that you can use in multi-color FISH in contrast to immunophenotyping, and basically, I

mean, one can easily look for amplification of HER-2, P-53 mutations, et cetera.

So I think that the quality of those cells may be very critical in determining how many you can have. I think it would vary tremendously between tumors, well, and between patients with the same tumor depending on what those genetic changes are and those phenotypic changes.

One will have to look at those and then correlate it with the subsequent course of the patient, I suppose, to get a definitive answer.

We're particularly interested in screening. I mean, we've done some bloods from patients with colon and lung cancer, and I think this test will work for all of the carcinomas.

I haven't discussed prognostication, but there's some major opportunities for a test that can prognosticate whether, for example, the cells that are circulating already represent metastatic cells that are destined for apoptosis, and again, I'm hoping the combination of cell count, of immunophenotyping, and of multi-color FISH will give us information which, in toto, will tell us or perhaps answer that or answer it at least for a proportion of the patients. It would be attractive

not to have to do mutilating operations on a patient who has a particular set of criteria where you can unambiguously say that patient has cancer.

I want to emphasize that. To replace the gold standard of biopsy and to act with treatment decisions on this, you need another gold standard. So it has to be rigorous. There can be no false positives. You can have some false negatives where you miss some here or there, but when you say this patient has cancer on the basis of a blood test, it has to be 100 percent.

It's not that difficult to do. You just have to examine every patient that comes in with a lump in the breast, get the criteria, and then code the sample, get an answer, and then see what criteria unambiguously mean cancer. The same thing can be done for all the other tumors.

It would be very attractive if one knew that a patient who had a mastectomy was cured. In the best of all worlds, if you found there were no cells after that in 90 percent of the patients and ten percent did have cells, and those ten percent went on eventually to relapse, you would save 90 percent of the patients from having the high dose adjuvant chemotherapy which they presently have,

which is disabling, and some of the disabling is irreversible.

So there are opportunities to do prognostication here that are attractive, but our first ambition, our first goal is to develop this as a screening method, and we have a long way to go because it has to be, as I told you, a gold standard, and I hope to reach that goal if I could ever get funding from the NCI.

(Laughter.)

DR. UHR: Thank you.

(Applause.)

DR. GREENBLATT: I apologize, but you know, emotions overcame me.

(Laughter.)

DR. GREENBLATT: Would someone give me the program so I can introduce the next group of speakers? I forgot to bring it up.

Okay. You're still on Thursday, Jay. This is -- we've moved ahead.

Okay. The next talk is by Dr. Dave Hoon, entitled "Detection of Occult Tumor Cells in Body Fluids and Lymphoid Tissues by a Multiple Marker PCR Assay," and Dr. Hoon comes from the John Wayne Cancer Institute.

DR. HOON: I'd like to thank the coordinators for this meeting and Raj for this excellent meeting. It has been very informative.

And in this session, what I've been told is to try to interpret my work in terms of microdiagnostic and what may be relative to assessing vaccines and actually addressing sources of potential cells for use in vaccines.

So what I will do is cover some of our work studying molecular diagnosis and try to interpret and to fit into the theme of this meeting.

Can I get the first slide?

So the talk is basically going to focus on occult tumor cells and different body compartments, and primarily I'm going to talk about solid tumors. That's where most of our experience is, particularly melanoma, breast cancer, and GI cancer.

Now, you can look at occult tumor cells using PCR or TPCR. I don't have to go through the procedure. I mean, most people are familiar with that, and basically you can look at different sites, organ sites, tissue in non-organs such as skin, tumor draining lymph nodes, fine aspirates and body fluids and blood, which is the most optimal, usually

the site which can be used repetitive assessment, peritoneal cavity, bone marrow aspirations, which is well, know, cerebral spinal fluids, and lymphatic drainage fluids from wounds you can use.

Now, to those skeptics, as Dr. Uhr has said, there are tumor cells in blood, and you can actually detect them. Often some people don't believe in that, but they are and you can find them.

Now, this is a slide that was shown earlier by Dr. Morton, but again, I want to emphasize this in more of a molecular term. The heterogeneity in melanoma, you can get different pigmentations which often interprets different levels of messenger RNA for the different pigments, such as tyrosinase, TRP-1, 2, GP-100.

Similarly, when you get metastasis, you can get changes in pigmentation, adaptation, and so the tumor is often continually evolving, and there's a continual genetic instability which interprets changes in tumor antigen expression, and this is important. It's an inherent problem, particularly when you're doing molecular diagnosis with particular markers.

And this just reiterates the point that you can have metastasis made up of many or primaries

made up of many clones, and metastasis does not necessarily, can reflect what the primary is, and you can get metastasis from a metastasis, whereby this is amelanotic and it can go adapt to a distant organ, and it can turn back into melanotic tumor.

So a tumor is constantly progressing, and it can change at different organ sites. There is no rule to say it will always maintain that particular phenotype, and that's one of the inherent problems in addressing therapy, and it's one of the inherent problems in vaccines.

And you saw this slide earlier from others. It showed that in melanin synthesis pathways, tyrosinase, TRP-1 and TRP-2, and for molecular markers, differentiation antigens for a particular tumor which is derived from a org. cell type that has particular markers you can use as good markers that are not found in normal cells, and in melanoma, this is where most of the work and a lot of detection of molecular diagnosis for occult tumor cells has been successful because the markers are very unique.

The markers are not totally unique in that you can find these myelogenesis markers, part of the dopamine cascade, which is found in retinoid,

and you can also find in the brain tissue and neural tissues. So it's not totally absolute, but in the compartment that you're testing in, such as lymph nodes, bone marrow or blood, it is not present.

So one of our concepts that we developed in the early '90s was using -- because of the heterogeneity problem, we addressed this by tumor marker heterogeneity. So we established the multiple marker concept. This is, in other words, using multiple markers to address the tumor marker heterogeneity, knowing that primary metastases or as the tumors continue to evolve, they are going to change. You have to address that that no single marker is always going to be present. So this is very important.

The other, which is often forgotten, is marker level of expression. You can have different levels of MRNA expression of a particular -- like, for example, tyrosinase which can vary from one melanoma cell to another cell, and that's a common factor that's often ignored.

So when you're looking at occult tumor cells, basically tumor cells, melanoma cells, for example, with a certain number of messenger RNA diluted in normal cells, you get a dilution factor

and which will affect your assay and how sensitive it is. So you also have to address that.

Another problem of single marker assays is you get false positives, which it often addressed.

The other is having a multiple marker, and these several markers are two marker at least positive, confidence in the assay, and it proves the assay sensitivity and specificity, and this is what our overall design of all of our systems has been based on.

These are some of the early markers that we use. Obviously there's more, but you can divide markers into melanoma, myelogenesis related, tyrosinase, TRP-1, TRP-2, GP-100, MART-1. You can do tumor progression markers, MUC-18, gangliocytes, and there's the tumor antigen markers. There are a lot more out there now. This list keeps on growing and growing, and they can be used as potential markers if they're screened through.

In our assay, it's a very simple assay. It's basically an 8 mL assay with sodium citrate-2. We collect the blood. RNA is extracted under SOP, and the quality control is done in the whole assay system.

I mean, the assay looks very simple, but if it is not done under rigorous conditions, you insert many types of errors and also contamination problems which will reflect your results. So SOPs are very -- especially in these sensitive assays -- are absolutely necessary, and the environment that they're conducted in.

And this is just one of our earlier studies I want to discuss that we had published several years ago where we used this set of markers, four markers, tyrosinase, P-97, melanin transferase, MAGE-3 and MUC-18, and this is in melanoma cell lines it's expressed frequently, but most markers often are over expressed in melanoma cell lines and often not representative of true biopsies. And in normal cells, they're negative except for MUC-18 where we find some.

Then we perform studies on different stages of patients and taking a blood sample, and categorizing the stage at the time of the blood sample, as you can see, there's a wide distribution.

There's heterogeneity in the markers, and at the same time they're more frequently in the advanced stages as expected.

And this is just to compare one marker versus two marker positive. We find at the advanced stages we usually have a greater number of two marker positives, and this is what we based the two marker cutoff.

And from our studies in this type of study we found the specificity about 95 percent and 96 percent for sensitivity, and since then we've actually adapted, changed this assay to a more refined assay. Actually you can get a much higher sensitivity and specificity now, refining our markers.

But in looking at those patients I just showed you, we looked at the Stage III NED patients. These are basically patients who are bled and had no evidence of disease at the time of blood draw, and then we followed these patients up for now over two and a half years, and basically we compared those with one marker or no marker positive versus two and four marker positive, and this is the current survival curve showing that the patients at two or four markers had a greater incidence of reoccurrence. It's about almost 75. Twenty-five percent reoccurred within one year, and this was significant.

There's also a multivariate analysis comparing the standard factors that are used in melanoma, the natural history of reoccurrence.

Similarly, in longer follow-up, the one marker or no marker did better versus the two of four markers, which is almost now 50 percent in reoccurrence of disease.

And these studies initially showed us that you can use these for prognostic advantages in looking at whether the potential of these patients for reoccurrence, and also they tell you does detection of tumor cells in blood in clinical free patients -- are they having any significance, and that's what we were addressing.

Also, the other thing that they also said is that looking at tumor cells in the blood and seeing it's positive, it also addresses whether you have sub -- we call it subclinical disease, and to prove that, as Dr. Uhr said earlier today, you have to verify it, and it does take some clinical follow-up to verify whether these are really going to be relevant, these tumor cells in the blood, and there has to be some standards in clinical trials, which currently we're doing this in a multi-institute international trial looking at bloods from patients

and being treated and following up for over five years.

Similarly, you also have to look at the clinical follow-up, and it has to be done very rigorously and well documented, and these patients were basically followed up every two months with rigorous analysis for reoccurrence of disease.

Another assay that we developed based on the three marker system using a semi-quantitative approach where we based on the number of marker positives and doing different dilutions of the blood, and basically to go over this quickly, a scale of zero to ten where ten is highly positive, basically very strongly positive, and zero is none.

In this system one of the best ways for identifying clinical disease is surgical staging, in other words, a surgical removal of the tumor to make the patient disease free and then looking at the blood before and after to really identify are you removing the disease and does it have an effect on the blood, and this is what we were doing pre and post surgery, and these are the types of stage of disease, and these are the size of the tumors.

As you can see, the larger the tumor, there was a significant decrease in the actual

values, and where the patient has a very small single node of metastasis, there was limited change, as you expect, but this is the type of studies that we're doing to validate the assay and looking at pre and post surgery.

Using the same assay for semi-quantitative and looking at different stages of disease, when you talk about stages of disease you have to say is there no evidence of disease or alive with disease and compare the two different categories, and you can see in the different stages that especially I and II and II, NED and AWD, there's a significant difference between the groups.

However, in the Stage IV, there isn't, and usually in Stage IV, as you know, they often reoccur very quickly. They usually have subclinical disease or smoldering disease that's usually present, and as you expect.

And these studies are ongoing. This is based on 75 patients, and now we are accruing more patients to really validate the significance of these findings.

Going into another, I talked about the myelogenesis markers. There are other markers, and here we use a carbohydrate marker, which was a tumor

antigen. It's originally defined by Dr. Ricco Erie, GM-2 and GD-2 as oncophetyl antigens in the early '80s, and in recent years the clinical utility of GM-2 has been shown by Phil Livington in terms of vaccines, and so we developed an assay to look at synthesis of GM-2/GD-2, what we call as basically the enzyme, and a set of galactose immune transferase.

And this, just to go over it quickly, this is just a comparison showing the blots. These are Southern blots, RTPCR and Southern blots. This is genomic. This is the actually PCR band, and it shows you the biopsies in patients who are positive, Stage III and IV, and controls.

And then what we did here is melanoma cell lines showing the different blots, and we did the actual ganglioside isolation biochemistry analysis showing GM-2 and GD-2 levels of cell lines and how it correlates to actually the MRNA level, and this is a correlation study.

And this just shows you what we did in looking at different stages of disease. As you can see, overall they are found in different stages of disease. However, the overall difference is not that significant, and so that they're there or

they're not there, and that led us to what's the advantage of this marker just as opposed to detection.

And then what we did was look at patients who had a certain level of disease and then follow them up over a year and looked at whether they had progressed or they didn't progress, and then looked at actually whether they were positive or negative.

And what we found is a significant correlation of those that were positive that reoccurred, clinical reoccurrence of disease in a short period of time. In other words, the patients who were GM-2 positive from their blood developed disease much faster than those who were negative, and so we call this as a potential progression marker that can be used to identify reoccurrence of disease.

Another site which is often used especially in breast cancer is bone marrow aspirations, and this, as was talked about in this meeting, is also a source for getting stem cells and dendritic cells.

And we know in bone marrow tumor cells occur quite frequently, especially in breast cancer

and in prostate cancer, but an unusual finding that we found actually four or five years ago, that in melanoma there are occult tumor cells in the bone marrow.

However, in general, metastasis to melanoma is less than ten to 15 percent, and bony metastasis rarely occur, but we find a great frequency, and other groups have also now found this, too, that there are more tumor cells actually present, occult tumor cells. Whether they were alive or dormant, we don't know, but it's very frequent.

Now, actually this study was not a bone marrow aspiration. This was actually a bone marrow biopsy. During thoracotomies for melanoma patients, that's what Dr. Morton was doing, and part of the procedure is to remove about an inch of the rib, and what we did was take and remove the rib and took out the marrow and then assessed for tumor cells. So this is actually a direct assessment of patients with metastasis in the lung undergoing thoracotomies.

And similarly, we've also now done with melanoma -- these are studies with Steve O'Day in our clinic -- and looking at bone marrow aspirates,

and we can use different markers, and we can show that there are actually tumor melanoma cells in the bone marrow.

And also studies have reported that colon cancer, which doesn't metastasize to the bone marrow, but actually there are occult tumor cells. So bone marrow may act as a sponge or actually as an indicator that metastasis has occurred, systemic metastasis has occurred at some point during that patient's life, evolution of their tumor, but when it occurs or not and somehow the tumor cells stay stable.

A lot of extensive studies have done immunocytochemistry, such as by Richard Codey, especially in the relevance of bone marrow metastasis in breast cancer.

Another study that we do, it was pioneered at John Wayne, is the sentinel lymph node study, lymphatic mapping. This is a study that was developed by Dr. Morton and his colleagues for identifying the first draining node which is likely to have metastasis.

And in this study what our objective was is to look at the detection of occult tumor cells in that lymph node, and what we devised is a plan where

we bivalve the node, section the node, using the frozen sections and with parallel section we do immunohistochemistry and HNE, and then do RTPCR, and this alternates to detect occult tumor cells.

In other words, this was a focused attack to detect occult tumor cells in the draining lymph node, and as you know, in melanoma and breast cancer, the draining lymph node positivity is a very important staging factor, and this is a question that we're addressing in one of our central mode randomized trial, is to determine what is the real value of actually occult tumor cells, and we still don't know.

In breast cancer, single occult tumor cells, we still don't know the total relevance. When we see micrometastases, it can be multiple cells or it can be single cells, and we still don't know what their relevance is still at this point until further studies are done.

But just to show us for our melanoma studies that you can use parallel melanoma markers that we've used, and you can see in all sorts of different levels of frequency showing the heterogeneity and the different levels, and these are actually HNE positive tumors. So these are well

defined metastases showing, and if you do IHC, in other words, histochemistry, to define occult tumors, this varies even more because you have a more dilution effect.

And this just shows you the seminal lymph node study.

So in other words, combining the sentinel lymph node study with molecular diagnosis provides one of the most focal attacks of trying to detect occult tumor cells, and the draining node is likely to have tumor cells, and this is ongoing studies in both melanoma and breast cancer.

And this is actually important as some of the issues individuals here have been discussing about using lymph nodes as a source, and one of the problems is having a few occult tumor cells -- really do mean anything, and still we don't really know, in other words, occult tumor cells that can be define by immunohistochemistry that maybe a few cells are those.

Obviously when you have well established micrometastasis by HNE, there is definitely major disease.

And so when we look at different sites as I showed, lymph nodes, bone marrows and blood,

one of the problems is in PCR you have to always address to the background expression of normal cells. Our sensitivity in melanoma, I always usually say one in five cells because you never can really tell one cell error in a limiting dilution. It's about one in five cells for melanoma in about 40 to 50 million because in melanoma you have a good, definitive marker.

In carcinomas you drop down to ten to 20 million and using a panel of multiple markers.

The other is you've got to have different stringencies. Obviously in the lymph node you're going to get more contamination of normal cells in there. It's always draining, especially in the breast cancer. You always get epithelial cells, normal draining into the lymph node. It's just a fact that occurs.

And specificity of the reaction, and final verification of the PC viral product and genomic contamination.

This is a study which I want to emphasize on some reports in the literature which we have also published. There are several markers out there that have used CEA, CK-19, MUC-1 as RTPCR markers. Although people consistently use these

markers, they have shown considerable false positives.

When you have greater than five to ten percent false positives, you have problems in your detection assays, and this just shows you -- I can give you the names of the list -- but these are from various publications that show the level of false positives just by RTPCR of these markers, and it's well demonstrated in the literature, and these are some other studies.

And so these markers for carcinomas are really not well established, and there's very few good markers out there for carcinomas for RTPCR because of this problem, and this is going to be a problem until new markers are really defined.

And the other problem with the markers is that when you establish a marker, you want consistency. As I said earlier, you have heterogeneity. There's no use having a marker that's only ten percent because you're only going to get ten percent. You're not going to pick up all of the tumors.

And when you're hunting for occult tumor cells from metastasis or not, your efficiency is not

going to be there. So you have to look at that very critically when you design this.

And epithelial cells, carcinomas, basically we still have a long way to go in this field.

Similarly, if you look at immunohistochemistry of breast cancer, there are still trials, and the American College of Surgeons is going to run a randomized study looking at a cocktail of antibodies looking at occult tumor cells soon to determine whether the efficacy of actually detection.

These are some of the markers that we use in breast cancer. I can show you the cell lines and tumors and bloods. These are 75 patients we looked at.

MAGE-3, any of the MAGE families are very good real tumor markers, solid, and they can be used very efficiently.

And this is beta hCG, which we also use. It also can be used quite well.

Factors influencing results, and there's multiple factors, and whenever you look at any of these PCR molecular assays, they always have to be

optimized and SOPs under rigorous control. Otherwise you bring in a lot of errors.

As I said, SOPs. This is a standard SOP. I'm sorry it's upside down, but this is our system that we run.

(Laughter.)

DR. HOON: And contamination, which is very important, and especially when you're doing this section. It has to be determined.

And the fusion of the two evolving technologies. Amplification technologies are constantly changing. The methods, the markers, and specificity are changing, and eventually we will have good assays that are of clinical pathological utility.

And these are some of our recent studies. Now we've converted away from gel electrophoresis and are now straight doing solution PCR using electroluminescence where we get more quantitative analysis and definitive. So you can basically now run the PCR for a patient and know within four to six hours from a sample. So no more gel electrophoresis. We've changed the whole system, and the least quantitation also.

One of the questions that was addressed for this symposium is what is the fate of occult tumor cells. The positive scenario is obviously the growth of tumors, and the other is metastasis at distal sites, and another is dormancy which can occur.

The negative scenario is natural inherent death, apoptosis, or most of the cells don't survive. Metastasis is basically very inefficient, and therefore, often none of the learning cells don't, and as we discussed in the last two days, immune regulation destroys tumors. So that plays a factor.

And lastly is the questions that need to be asked. What is the role of occult tumor cells detected by immunohistochemistry? We still don't know this fully, and there are still trials out there.

And the same thing. Until we know this, this is still going to be difficult to answer. So there are still some questions to be answered.

Thank you.

(Applause.)

DR. GREENBLATT: Thank you for your talk.

I just wanted to introduce our last speaker, Dr. Carleton Stewart, and he will be talking on detection of cancer cells in bone marrow by high speed cell sorting.

Dr. Stewart.

DR. STEWART: Thank you.

Well, first I'd like to thank the organizers for inviting me to this really what I call a very relevant meeting, not only relevant for what we're facing now, but relevant for me, in particular, because we're monitoring a lot of the very tests that have been discussed here, and I'd like to do it right.

Now I can assure myself that nobody knows what right is.

(Laughter.)

DR. STEWART: But basically what we're looking at about four years ago our approach or our challenge was to try to monitor hematopoietic products that are going to be used for autologous transplants in breast cancer patients, and we started out to do that, and we didn't think about using iron particles with the antibodies attached to them because that's a very inexpensive way to do it

compared to spend a half a million dollars for a high speed cell sorter.

Fortunately, we have other applications for the high speed cell sorter, and so the first thing that we see is this is what we see in a microscope. Is there a tumor cell in this field?

And with a fluorescent marker we can find those tumor cells or we can find cells that masquerade as tumor cells, and as we've heard, 100 percent positivity, that is, we have to be positive that it's a cancer cell and not a cell masquerading as a cancer cell when we look at this cell.

And flow cytometry allows us to do two things. It allows us to put multiple markers together so that we can get better sensitivity and specificity, and the only way we're going to get sensitivity is to run enough cells.

And so a high speed sorter can process 150 million cells an hour, and we can sort those cells on a microscope slide, and if you had one in a million, that means you can sort 100 of those cells on a microscope slide and use independent technology to confirm, as we've already seen from Dr. Uhr's talk, that that is a tumor cell.

And here we see a cancer patient in which we looked at the blood of that cancer patient, and you can see that there are no tumor cells. There are no cytokeratin positive cells in this region.

Here's CD-45 versus cytokeratin, but when we look at the bone marrow of this very same person at the very same time, we see a very high frequency of tumor cells.

So the first message is that they may accumulate in the bone marrow, but they may be moving by quickly in the blood, and so the bone marrow could be a much more sensitive place for detection of these cells, but certainly we probably wouldn't want to give this preparation back to the patient.

Now, in comparing the original tumor, and this is often one of our -- is to be able to phenotype the original tumor, the primary tumor from the breast cancer patient and then look because now you know what markers they express, and use that to develop individual, specific cocktails for finding these cells.

And here we see that in the original tumor in this case DNA is the only marker. The bone

marrow has exactly the same DNA content as the original tumor, and this bone marrow was taken almost a year later after the primary surgery resection on this cancer patient.

And so looking at our experience up through about 1997, we see a couple of things. First of all, there were 21 patients that we called negative or indeterminate for whether there were circulating tumor cells.

Now, remember we're measuring 100 million cells here, and you could see that normal, healthy donors like me -- huh, some people think I'm health; some don't -- there were 906 breast cancer cells in males and females who were healthy, right? That is sensitivity, but not specificity.

Those are not cancer cells. They're not even epithelial cells. It isn't the result of sticking a needle into your arm to get the blood. I'll tell you what it is a result of in a second, but they're not tumor cells.

And here we have patients which have a lower frequency in the 21 patients here than a normal, healthy donor does.

Now, here we see the matched health donor with patients in which there are more tumor

cells or there are more cells in the eight tumor patients than there are in the healthy donors. Are these tumor cells? Just because there's a difference, a mathematical difference, is that tumor cells? Is that tumor cells? Is that specificity or is that by chance? I mean seven out of 21 patients.

I think you'll agree probably this one might have contaminating tumor cells, but I don't know what the other ones have.

And so we set out to find out what cells are in normal, healthy people that meet the criteria of being a cytokeratin positive, CD-45 negative cell, and it's a lot of fun because the world of rare events is almost as much fun as the world of lots of events.

And so the first thing we need to do is develop an assay that not only is sensitive, but is also specific, and so we have some definitions here.

First of all, criteria regions are Boolean combinations of regions designed to resolve cells of interest. Does that means cells of interest are really the cells we're interested in? Maybe.

Events are particles that are acquired by a flow cytometer. Did I say "cells"? I said "particles."

Positive events are events that are above some marker we set that we call positive. They're positive for that marker, but what does that mean? What is being positive for a marker?

Now, positive cells -- there's a different question -- are the cells that actually express the epitope for the antibody.

Specificity is the frequency or percentage of positive events in the criteria region, events, not cells. But specificity is the frequency of positive cells, the real cell, in the criteria region, and this is the only thing we want to measure.

Here we see a blood specimen in which we have stained the cells with a combination of CD-32, 41, 45, and CD-105, and you can see that in this combination we have MUC-1 and we have ERB B-2, and we have cytokeratin.

Now, if we collect this many events, we're pretty happy. We didn't collect enough events to find out if there's any cells masquerading in this normal blood. You've got to collect enough to

see them, and so we are going to set a region in which we are going to collect cytokeratin positive cells.

And you can see here that this is a normal person which has 900-some odd masquerading tumor cells per million, but if now we say that these cells also have to be MUC-1 positive and ERB B-2 positive, and we call this combination of antibodies here our heme combination, we now see there are no cells per million that are in our criteria regions.

Now, I don't have enough time to show you all of the evidence, but basically what we did was to sort each of the populations and identify them that were contaminating from a healthy donor the criteria regions.

We found they were eosinophils, eosinophils. What are eosinophils doing CD-45 negative? Well, that's a whole other question I can write an NIH grant for, right? And you're all going to give me the money. A very important question, CD-45 negative eosinophils. But they are CD-32 positive.

What about CD-41, micro megakaryocytes? I don't know if they're cytokeratin positive or not,

but they are positive events that contaminate the cytokeratin window, and we can get rid of them by simply adding CD-41, and when you sort them, I call them the Spidermen of your blood, because they have these gorgeous nuclei that stain blue with Herkst (phonetic) and they look just like Spiderman outfit, as the platelets that have not yet fragmented. And every single person in this room has them circulating right now.

And a third are endothelial cells. In fact, I told a colleague of mine about this, and we sorted some, and she's growing them in vitro, in culture now, right from your blood, endothelials. Just take them out of your arm and put them in culture. You can grow your own endothelial cells. You don't have to pay all of this money for this cocktail to grow the ones from the repositories. You can get them right out of your own arm.

(Laughter.)

DR. STEWART: Now, we look at just a proof of principle. We contaminate this preparation of blood with a tumor cell line, which happens to be cytokeratin positive, MUC-1 positive, and ERB B-2 positive. Oh, wouldn't it be wonderful if everybody was like that? It always works with cell lines.

Make the biggest claims with the least amount of data.

And there are the cytokeratin positive tumor cells. If we -- oops, I think it's tired -- and you can see now that we are seeing our cytokeratin positive cells here, but are there any contaminating blood cells?

Well, we know there are because I showed you the specimen we didn't contaminate, but again, if we now go to our most stringent criteria where they have to be negative for this, positive for cytokeratin, MUC-1 and ERB B-2, the data that we get by adding the number added and the number we found, which is this one, which is the highest specificity, we have recovered virtually all of the tumor cells without any contamination at one to a million.

So are there two per million? Well, let's see. Maybe.

And here's a normal and floor patients. These are the actual ones. If we looked at cytokeratin, we have 1,778 tumor cells in this normal person. I don't think so.

And we see that these patients have lie numbers (phonetic). If we now take our HEEM negative cells, we now add that to our criteria. We

now have one in 12,000 or one in 13,000 that still meet that criteria.

If we add MUC-1, we have now one in 168,000. That's our specificity, and if we now add ERB B-2 so that we now have one, two, three tumor markers, we now have greater than one -- we have less than one in a million masquerading cells in our blood, and yet we can still see that we can detect significant numbers of cells in the patient except for Patient 4, which we could not detect any tumor cells.

So with high speed sorting then, we can interrogate 100 million cells in 55 minutes and sort in this example 746 cells onto a microscope slide for further study.

Thank you.

(Applause.)

DR. GREENBLATT: Thank you.

I'd like to thank all of the speakers of this session and all of the speakers we've had over the last two days, and could those who are participating in the panel discussion come up to the podium?

DR. RAZZAQUE: I would like to start discussion on this session, Session V. On page 56

of the program book, you will find two sets of questions. The first set of questions are on detection and characterization of contaminating tumor cells in cell vaccines.

The second set of questions are on dose selection for irradiation of tumor cells to be used as vaccines.

You know that tumor cells are sent into the peripheral blood and may reside in the bone marrow. So when you use these cells, PBMCs and bone marrow, for generating cell vaccines, for example, dendritic cells as you have heard in this two-day meeting, there is a chance that you might pick up contaminating tumor cells.

To insure the safety of these kinds of vaccines, cell vaccines, we have formulated some questions, and we need to address these questions in this session.

Dr. Uhr has shown a method of detection of cancer cells in blood, and he has detected one in ten to the eighth cells in leukocytes by immunomagnetic amplification, flow cytometry, et cetera.

Dr. Stewart has shown a high speed cell sorting method of detection of cells, and Dr. Hoon

has shown PCR method of detection of contaminating tumor cells.

With that I would like to ask the first question to the panel and to the audience to be addressed. The first question is: what would constitute acceptable methods for identification, quantitation, and characterization?

Panel, please.

DR. UHR: I think it might be in the future -- let me just say this. I can't answer any of these questions, and I don't think they're answerable right now.

But I think in the future one might well be using all three, a combination of all three of the techniques we're discussing. One is going to have to be able to count the cells, and one is going to have to characterize them in great depth, both in terms of the proteins they make and in terms of their genetic alterations.

I mean, as a simple example, if you find a certain number of cells, X number of cells in the bone marrow after purging, and these cells have amplification of HER-2; they make a lot of Cyclin-D; they have some replication, invasive markers, et cetera. One would speculate that one might have to

remove more of those than if you have the same number or smaller number of cells which don't have HER-2 amplification, which lack many of the markers, let's say, that we associate with aggressiveness in terms of tumor cells.

In fact, what if none of them are dividing, or some of them have some of the changes of apoptosis, membrane flipping, for example?

So it seems to me that it's going to be different with different tumors and among different patients, and there will be heterogeneity even within the cells of the same patients, as has been carefully brought out.

So you'll want a technique where you could look at a lot of cells quickly. Whether it's slide based or through the flow cytometer, I don't know because they keep on improving both, and you might well want to do some PCRs to pick up particular mutations which can't be done by multi-color FISH on interface cells, et cetera.

So I think one might well not pick out a technique now, but say that we may well have to use all of the advances that take place from these three different approaches.

DR. RAZZAQUE: Dr. Stewart, please.

DR. STEWART: Yeah, I certainly agree with that. The techniques we use are going to be the most difficult thing to answer, but in my opinion there's only one question, and it's a tough question.

The only thing we have to know is if the tumor cell that we see contaminating the bone marrow or the blood or wherever is clonogenic because I don't really care how many tumor cells there are in this patient's bone marrow if there are in any of them clonogenic.

And so the challenge we have is to develop a reliable assay for clonogenicity, and it may, indeed, include all of the assays that Dr. Uhr just mentioned, but that is our challenge, to determine and select a clonogenic tumor cell.

And I think Dr. Hoon's data where he sees melanoma cells in the bone marrow and there aren't any melanomas growing in the bone marrow is an example of that, and we have lots of examples of that, not just that one.

DR. UHR: Can I just mention I'd like to throw in an addendum to that? I don't like to disagree with Dr. Stewart. I enjoyed his presentation too much.

(Laughter.)

DR. UHR: But tumor dormancy is a problem. I mean I personally think cancer is a chronic disease. It disseminates very early, and then after you, quote, cure the patient, there are still the cells that are around. We know that's classical for melanoma and for breast cancer. They come back as late as four decades later. But I think it's true for many of the other cancers, perhaps all of them.

So the fact that you might have a clonogenic cell doesn't in itself definitively indicate that you are going to have in vivo, in this particular micro environment, growth or that the growth may not be balanced by cell death.

For example, in the mouse model that we use, most of the cells are in cell cycle arrest. There is a population of a million cells in the spleen, and they're dormant. The animals are heavily immunized, and most of them are in cell cycle arrest, but a subset are dividing and apparently dying at the same rate by apoptosis because they carry the same one million cells throughout their life.

I don't know whether this is going to be the same in the human or not, but I think clonogenicity would be worrisome, but it wouldn't necessarily equate with the fact that these will not be dormant.

DR. RAZZAQUE: Dr. Stewart, do you want to comment on that?

DR. STEWART: I would like to respond to that quickly.

DR. RAZZAQUE: Yes.

DR. STEWART: And that is my definition of clonogenicity does include dormancy. A clonogenic cell could very well be dormant. We have to find the assays that are going to measure a clonogenic cell, not what it's doing right now, but a clonogenic cell.

And I want to make that because it's important. Just because they're proliferating doesn't mean they're clonogenic. Just because they're dormant doesn't mean they're not clonogenic.

DR. UHR: I agree.

DR. RAZZAQUE: Dr. Uhr, I have a question to your opinion to your experiment. Are you getting -- this method that you are using for

epithelial cells, is this applicable to other types of cells, like hematopoietic cells?

DR. UHR: I think it may well be applicable. That will be much more of a challenge. I mean, the epithelial cell, coming from the ectoderm, and the blood elements from the mesoderm obviously have different genetic programs. So there's just a plethora of markers on the cell surface and intracellularly which distinguish them, and that's why we're able to get down to this sensitivity.

Now, when you begin to deal with other, let's say, hematopoietic tumors, I mean, that's going to be more of a challenge. You're going to have to look for quantitative differences, et cetera, plus mutational differences.

I think it may be solvable, but I'm not the one who's going to do it. It's a tough job.

DR. RAZZAQUE: You need more money from NCI.

DR. UHR: No, I can't add anything to what has already been said.

DR. RAZZAQUE: Dr. Hoon, please, any comment?

DR. HOON: I agree with the other two panel members.

One of the factors it is is the source of actually where you're going to harvest the cells from which will be critical. If you're going to do it from the lymph node, the gold standard will be immunohistochemistry, which is acceptable by others.

Blood, it will be a little bit different, and bone marrow aspirations for breast cancer patients, immunohistochemistry is used.

However, the other aspect of it is what level of disease. Most of the studies that have been presented are giving vaccines to patients with tumor burden, and the micrometastasis or so-called occult tumors I don't think are going to really affect overall what the influence of the overall if they do contaminate and grow, especially in patients with tumor burden already.

The patients who are disease free or earlier stages where you are giving vaccine and you are giving tumor cells, there are some studies out there that do affect, but those require clinical follow-up in order to really determine that.

So those patients who are early stage or have no evidence of disease, it will be a critical

factor, but advanced patients, we are probably pushing occult tumor cell's detection a little bit too far.

DR. RAZZAQUE: Dr. Hoon, I have a question. Your PCR -- okay. Address first, please. Okay.

DR. STEWART: Can I ask a question? These methods are extremely important for early detection, for prognosis, for monitoring efficacy of disease, but can someone define the knowledge base for the danger of taking cells out of the body to make dendritic cells, taking that out of the blood, adding GM-CSF and IL-4, and then putting back the same tumor cells that you got from the blood, except now you have them in an environment where you now have dendritic cells there where you didn't before?

You know, maybe I'm very naive, but we're just making this assumption that we have to deal with this situation, and I want to know what the knowledge base is that it's a dangerous situation. Like has this been done in animal models where there's circulating tumor cells and, you know, you make dendritic cells, put them back, and the mice get tumors or any evidence in humans?

DR. MARTI: That's a major concern. These not necessarily grafts, but these products, if they're going to be derived from an individual that has circulating tumor cells, we're seeking input on that. Do those cells need to be purged? If so, how? And what will be the lower limit?

And a subsequent question is: can you irradiate this product? And if you can irradiate it, how much?

DR. STEWART: I'm just asking the very first question you mentioned. Do those cells have to be purged? That's what I'm trying to get past.

DR. MARTI: Well, certainly from the stem cell labeling studies in pediatrics, the cells that were still contaminating the graft are the ones in the majority of patients that give rise to the relapse of the leukemia.

DR. LOTZE: Actually it's neuroblastoma studies you're talking about?

DR. MARTI: I'm sorry. I stand corrected.

DR. LOTZE: These are from Malcolm Brenner, and in actual fact I think the best answer to Jeff's question may be situations not where you transfer back into the same individual, but the

experiments where people actually transplant them into another human being, which occurs in the setting of human transplantation.

DR. STEWART: But that's totally different. That I understand. I'm talking about the same patient.

DR. LOTZE: But that is the fear, and I think Dr. Uhr's comment was relevant in the sense that maybe all of the patients we see already have at the time of detection disseminated tumor, and so the issue is will it transfer back into somebody who's nominally cured or seemingly cured of disease; transfer back into that individual of a fixed tumor inoculum increase their likelihood if those cells are viable, and to use the term that's been bandied around, clonogenic. Does that decrease their likelihood of being alive ten, 20, 30, 40 years later?

That's the question I think you're asking, Jeff, and for that there is no data. I mean the only data that exists is in the setting of Human A transferred into Human B because you don't know what's going to happen.

DR. STEWART: That's another story.

DR. LOTZE: But can I bring up an anecdote? Because I think it's an issue that's confronted us before, and if you'll allow me, I had a young woman in her late 30s who had widespread metastatic colo-rectal cancer who had two identical triplets. She is one of identical triplets, and I wanted to immunize one of her triplets to her tumors, and I pulled a variety of different oncologists and scientists around the world, none of whom felt it was ethical for me to do that because of the concern associated with potentially giving viable tumor to an identical litter mate, if you will, in whom my assuredness of knowing that that tumor was not going to grow in that individual could not be 100 percent.

So I said, "Well, what if it's a really, really small chance?"

Still the ethical issues prevailed, and so I think the question that Jeff asked is an important one because if you play this movie ahead frame by frame, I think what we're going to end up doing if we are successful is using some kind of therapy in which DCs are charged with early lesions or tumors derived from early lesions as a therapy.

And I think the question that was raised is: do we need to know that those tumor cells are not viable? Do we need to know that they're not capable of limiting someone's survival? And is the tradeoff, meaning the opportunity of potentially doing benefit, as opposed to the intrinsic risk worth it?

And somehow we're going to have to breach these kinds of issues. So I think the FDA is to be congratulated to ask the questions. We're going to test in people with advanced disease first. So I agree with the notion that it doesn't matter so much for people who have got ten other lesions in their liver.

But when we move it into a setting, which would be nice if we ever get to that point, where we can treat the early breast cancer lesion or the early colo-rectal lesion with an autologous vaccine, then those questions will become, I think, terribly germane.

DR. MARTI: I think you're right, Mike, but I think the most important thing is that we're able to measure them now, and we'll just have to wait for the clinical follow-up.

DR. LOTZE: Yeah, I think we'll get some information in the setting where we treat patients with early stage disease or patients who are very likely to recur, and I think we should have the courage to try and do something to help that group of patients and shouldn't limit us in terms of our ability to use these kinds of therapies.

But I think somehow knowing whether cells are viable or not or capable of modulating someone's long-term outcome is going to be a critical question.

DR. STEWART: There's another corollary to what you're bringing up to, and that is what is a reasonable sample size of what you're going to reinject back into the patient. Is it the entire specimen that you have to process to find out if there's any tumor cells there, in which case it's lost to the injection back into the patient?

Because you're not going to find tumor cells if the frequency that you measure is too low and the one that you look for is in the one that you're injecting into the patient.

Now, one thing about flow cytometry is that you could process the entire specimen and collect it instead of throwing it out, and you would

interrogate the entire specimen and know whether the tumor cells are there, and you'd have a marker for clonogenicity, right?

So you could say this one is clonogenic, and that one isn't. So we'll just sort it out of the way so that it's not there anymore, and there you are.

DR. LOTZE: It sounds costly.

(Laughter.)

DR. RAZZAQUE: A question there?

DR. KUZNETSON: Dr. Uhr, you can detect very low frequency of tumor cells in blood, and what technique allows you to increase the detection of frequency of tumor cells up to ten in minus eight?

Because I think there is some contradiction between your prediction, your estimation, and Dr. Stewart's estimation. You have two order differences between your estimations, if I understand.

And my question is: how many antibodies are used for detection of the cells? This is my first question.

And I do have some comparison analysis of new technique with previous one which you used in BCL-1 lymphoma when you detect dormancy or very low

frequency cells with PCR -- PCR reaction or using monoclonal antibodies against idiotopic antigens.

DR. RAZZAQUE: Dr. Stewart or Dr. Uhr, please.

DR. STEWART: Do you want to do that first?

DR. RAZZAQUE: Yes.

DR. STEWART: Go ahead.

DR. UHR: Well, I think the difference is the immunomagnetic purification. In the beginning I stressed to you that this was a critical step, and two of the known variables are the beads and their size. You just can't allow any clumping or one has a problem.

And the second one I mentioned was the anti-epithelial or the adhesion antibody, and that's absolutely critical, and it's a little bizarre, but anyone who works with even big beads will tell you that for reasons not clear, some antibodies work very well and others don't.

So, for example, the one that we're using, which was first described by Dorothea Hurlin, is excellent. We get 100 percent recovery, and we get that 10,000-fold purification before flow

cytometry, which is essential. That's ten to the fourth already.

Now, we've used another antibody which we got from another company I won't mention where basically we get ten percent recovery, and not only that; we don't get good purification, which is very bizarre. I don't want to spend too much time. You'd think they'd go inversely, but we lose at two levels.

So I think it's the very careful selection of the right anti-EPCAN antibody and the kind of beads, and I mean, this isn't something we worked out in one week. I mean a postdoctoral fellow spent a year trying to get this down.

But I don't know that there's that much difference. From what I understood from Dr. Stewart, it didn't sound as though we have a two log difference at all, and remember we still can get some cells in our normals.

I mean to reach the full sensitivity, the gold standard I talked about, we're going to have to show that those are not tumor cells and they are events, you know. We're going to have to show even if they're epithelial cells that they're not

tumor cells to be able to say that we can detect one tumor cell in 30 mL of blood.

DR. RAZZAQUE: Dr. Stewart, briefly please.

DR. STEWART: I'd like to say that the one really important advantage with the iron technique is right up front you get rid of a lot of noise. I mean you are already starting with a preparation in which all these other cells that are bothering me because I don't do it are gone.

That is a tremendous advantage because whenever you add multiple markers, you increase specificity because the probability of noise being coincident in a multi-parameter sense goes away factorially, and when you start out with getting rid of ten to the fourth units of your noise right up front, that is impressive.

DR. RAZZAQUE: Are there anymore comments from the panel?

DR. KUZNETSON: I agree with you this is a really effective way. If you've used few markers simultaneously on the same system, you can increase dramatically the sensitivity, but what about robustness of these measurements? Because you have a very sensitive system, and this system could be

not so stable, and it could have unstable detection. If you repeat the detection, you can get dramatic differences in amplitude of your measurements and frequency.

Do you have some observation about --

DR. STEWART: I don't think I'm following where you -- I don't understand what you're saying.

DR. KUZNETSON: I said about robustness of measurements and reproducibility of measurements. If you repeat the same measurements with the same object after a few minutes or a few hours or a few days, what happens if you repeat measurements and how many cells you will detect with this very sensitive method?

DR. RAZZAQUE: I would like to say that I would like to end this session. Can you ask him after this session this question specially?

I would like to thank the panel for your available discussions, and I would like to give this audience to Dr. Noguchi for concluding remarks.

(Applause.)

DR. NOGUCHI: For all of the hardy survivors here, which I don't know if you're

clonogenic or not, but I do want to thank everyone who has participated.

Cancer, in general, is a dreadful disease, and it's well shown by over half of the INDs submitted to FDA are for cancer, and in fact, that reflects the importance of this whole conference.

I would like to say that we've learned a lot. I commend everyone for the courage in being able to try to move forward on both the clinical trials, as well as the basic studies, and as we've heard, we seem to be kind of at the beginning of developing appropriate potency assays, as well as monitoring assays.

But I do have to say I very much appreciated this last session. Most of my training is in pathology and looking at tumors, and so I agree if you could see it, it makes a lot more sense to me than an adverse immune response.

I thank all of you for attending here. We will be making some decision on the recommendations and discussion that is being done here. If you really like this sort of thing, I think we might want to do it again, although I can tell you that Dr. Puri and Dr. Razzaque can tell you

that they'll be very appreciative if somebody else hosts this meeting.

(Laughter.)

DR. NOGUCHI: But it is very important for all of us in all of these fields to continue to get together because I think it's only when we apply all of the modern technology, all of the best minds that we can ever make progress in this deadly disease.

Thank you all very much.

(Whereupon, at 5:29 p.m., the workshop was concluded.)