FOOD AND DRUG ADMINISTRATION
NATIONAL CANCER INSTITUTE

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WORKSHOP ON TUMOR VACCINES

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THURSDAY,
DECEMBER 10, 1998

The workshop was held at 8:30 a.m. in the Masur Auditorium, Building 10, National Institutes of Health, Bethesda, Maryland

Speakers:

Allen Albright, Ph.D.
Jacques Banchereau, Ph.D.
Donna K. Chandler, Ph.D.
David M. Essayan, M.D.
Patricia Keegan, M.D.
Donald W. Kufe, M.D.
Larry Kwak, M.D., Ph.D.
Edison Liu, M.D.
Michael Lotze, M.D.
Gerald Marti, M.D., Ph.D.
James Mulé
Drew Pardoll, M.D.
Raj K. Puri, M.D., Ph.D.
Glenn Rice, Ph.D.
Ralph Steinman, M.D.
David L. Urdal, Ph.D.
Kathryn Zoon, Ph.D.

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P R O C E E D I N G S

(8:30 a.m.)

DR. PURI: Good morning and welcome to the FDA and NCI workshop on Tumor Vaccines. My name is Raj Puri and I'm the scientific and the clinical review and Chief, Laboratory of Molecular Tumor Biology in the Division of Cellular and Gene Therapy, Center for Biologics Evaluation and Research, Food and Drug Administration.

Before we begin, I'm required to say to you some of the housekeeping rules. No standing or sitting is allowed in the aisles by order of the fire department. There are two fire exists, one to the left of the stage which empties out in the front of the NIH Library and the other through the main doors in the back.

Complimentary refreshment breaks will be provided each day of the meeting. The refreshment station will be set up in the main lobby area of Building 10, not far from the Masur Auditorium.

Due to the amount of traffic throughout the building, we ask that you please wear your name tag as conference identification when participating in the refreshment breaks. Signs are posted.

Please note that no drinking or eating is allowed in the auditorium.

Now I would like to take this opportunity to thank our distinguished hosts, Dr. Zoon, Dr. Seigal and Dr. Ed Liu and our distinguished invited speakers, panel discussion participants, poster presenters and of course, the audience, for taking time off from your very busy schedules to participate in the discussion of some of the important old and some of the new issues in the tumor vaccines.

According to the latest Pharmaceutical Manufacturers Association Biotechnology News, there are 350 new biotechnology medicines in development. Among 350, 171 are for cancer. Seventy-seven vaccines are in development and a significant proportion of them are therapeutic cancer vaccines. Thus, there is a tremendous interest to cancer vaccines and other product development for cancer.

It is our hope that at this workshop we will hammer out some of the important deficiencies that might be impeding the rapid progress of tumor vaccine product and clinical development.

I would now like to introduce our first and highly distinguished speaker, Dr. Kathryn Zoon,

Director of the Center for Biologics Evaluation and Research, one of the six centers of the Food and Drug Administration. The Center is responsible for assuring the safety, purity, potency and effectiveness of biological products used for prevention, diagnosis and treatment of disease. Towards this goal, CBER conducts vital scientific research, establishes written and physical standards, regulates the testing of investigation of products, evaluates applications, licensing biological products, performs post-marketing surveillance and insures the continuing safety and efficacy through compliance activities.

Dr. Zoon brings to her duties as Director a distinguished scientific career. After receiving her Ph.D. in Biochemistry in 1975 from Johns Hopkins University, Dr. Zoon was awarded a post-doctoral fellowship at the NIH by Nobel Laureate Dr. Christian Envincent where she pioneered work on the purification and characterization of human interferons.

In 1980, she joined the Food and Drug Administration and continued her scientific research career as a senior investigator, as a Director of

Division of Cytokine Biology and in 1992, assumed the Directorship of the Center.

Dr. Zoon continue to conduct innovative research in the field of interferon purification and characterization. Dr. Zoon is the editor of Journal of Interferon Research and has received numerous awards including the meritorious executive rank award for Sustained Superior Performance in revitalizing and reorganization the Center for Biologics, Evaluation and Research to meet the challenges of new responsibilities and goals.

It gives me great pleasure to introduce Dr. Zoon who will develop welcome remarks and present conference goals and objectives.

Dr. Zoon?

DR. ZOON: Good morning. First of all, I'd like to welcome everyone to this workshop on tumor vaccines. I was reflecting just before the meeting, this is not a new area. We've been working on

tumor vaccines ever since I came to the FDA in 1980. However, I have to say the science has evolved rapidly and as witnessed today by the attendance to this workshop which you may by looking around you see as close to 500 people is quite impressive with

both the scientific interest as well as the progress we have made in this area.

I would especially like to thank the organizers of this workshop. This has been a tremendous effort to put this together, bringing all the right people to bear on the issues that we need to discuss today has been a monumental job and has taken the cooperation and really dedication of all involved in organizing and I would especially like to recognize Dr. Raj Puri for leading this effort.

We look forward to working with the NCI, not only on this conference, but many in the future as they relate to new medicines for the treatment of cancer. This is one area that I think offers amazing promise and should be explored together as a combined effort to make sure that the scientific standards by which we review these products are appropriate.

Recent advances in the identification and cloning and characterization of tumor-associated antigens and the isolation and expansion of potent antigen-presenting cells such as dendritic cells has generated renewed scientific interest in the development of tumor vaccines. A number of clinical

protocols have advanced to Phase 3 stages of development.

Although methods for characterization of cloned antigens or synthesized peptides are available, methods for physiochemical and functional characterization of whole cell vaccines, tumor cell lysates, polyvalent tumor antigen preparations, antigen presenting cells and other cell-derived vaccines are not well defined. For all biological products, general regulatory principles apply. For these products safety, purity, potency and efficacy will be needed for licensure.

Some of the above-mentioned products present unique challenges in terms of characterization of identity, purity, potency as compared to some other biological products. For autologous tumor cells such as vaccines safety, identification of major cellular components, and potency tests have not been formalized. The consensus on the appropriate immunophenotypic and functional characteristics of dendritic cells has not been reached.

Moreover, significant clinical issues remain to be addressed in the area of biological

immunological assessments as well as other areas in terms of their clinical applications.

Optimization and standardization are needed to develop acceptable immunological outcomes to guide the conduct of later phases of clinical trial development.

Prior to proceeding to randomize Phase 3 trials, particularly in the adjuvant setting, it is appropriate to require evidence of clinical activity or what effects on immunological surrogate endpoints be sufficient.

The FDA and the National Cancer Institute share a common goal to promote development of safe and effective therapies for human cancer in a timely manner. This conference is designed to address several major points. First, to bring together scientists, clinicians and regulators developing or helping to develop cancer vaccines products with the objective of being able to reach an understanding of issues involved in the characterization of tumor vaccines, the need for acceptable identity potency tests and clinical endpoints.

Second, identify when these issues need to be addressed in product development. Third,

provide a forum for open exchange of ideas and product development for cancer vaccines and in the design of clinical trials and to understand the FDA regulatory process.

I personally look forward to the outcomes of this conference. I'm sure they will be extremely helpful in providing the framework for the future licensure of these tumor vaccines.

Thank you very much.

(Applause.)

DR. PURI: Thank you, Dr. Zoon for very nice comments. I'm delighted to introduce our next distinguished speaker, Dr. Edison T. Liu, Director of Division of Clinical Sciences of the National Cancer Institute.

Dr. Liu is responsible for directing the internal clinical programs in cancer. Dr. Liu obtained his college and medical degrees from Stanford University. Prior to being recruited as Director, Dr. Liu served as a member of NCI Board of Scientific Advisors.

In 1995, he was named Chief, Division of Medical Genetics at the School of Medicine, University of North Carolina at Chapel Hill, where

he was a professor in the Departments of Medicine, Epidemiology and Biochemistry.

From 1993 until 1996, Dr. Liu was Director of NCI Designated Specialized Programs of Research Excellence in Breast Cancer.

Dr. Liu possesses excellent scientific career and has contributed more than 80 papers in the literature.

He's a member of numerous national societies and serves as Associate Editor for Breast Cancer Treatment and Research and Clinical Cancer Research.

He's an editorial board member of journals named Breast, Leukemia and Journal of Clinical Oncology.

In 1996, Dr. Liu received Susan G. Komen Breast Cancer Foundation Award for breast cancer research. This breast cancer research award was granted to him on his studies on signaling molecules involved in breast cancer and leukemia.

This morning, Dr. Liu will deliver keynote address for this workshop.

Dr. Liu, please.

DR. LIU: I want to welcome all of you to the Bethesda campus of the NIH and I must say

that I look very much forward to the proceedings of today's meetings since we all encounter the same issues and same problems in the intramural program in our vaccine development.

I must state from the outset that quite boldly that I am not an immunologist and I profess to have assiduously avoided tumor immunology for most of my scientific career. I'll admit to you that as a medical student I approached Reut's textbook in Immunology with the same foreboding as reading Joseph Conrad's Heart of Darkness.

(Laughter.)

Thus, I viewed this very kind invitation by the organizers and by Raj to deliver this keynote address for this workshop on tumor vaccines with a mixture of surprise and amusement. However, after some discussion with him, I surmised that what the organizers sought were thoughts from an impartial, but interested observer, not unlike de Tocqueville as he rummaged through the post-Revolutionary War in America or even Walter Cronkite commenting on the space program.

Viewed in this light, I found the challenge too tantalizing to refuse. We live in truly remarkable times. Our knowledge of

immunologic fundamentals, coupled with the ability to measure biologic molecules at picomolar concentrations and to produce precise molecularly agents as well as to amplify specific immune cells are making tumor vaccines a clinical reality.

However, your very success has uncovered a unique set of clinical problems rarely seen in the development of standard cancer therapeutics. I have full confidence that your immunological community will solve the basic scientific questions pertinent to tumor vaccines because the standards are clear and there is a consensus as to what is acceptable scientific process to address these questions.

However, the challenge is in moving these mature basic concepts and immunologic reagents into clinical testing. Unless these problems are properly and carefully addressed,the promise of optimal immunologic therapies reaching cancer patients will be forestalled.

In my recent sojourn through the field of tumor vaccines I have been struck by several observations that distinguish vaccine development from the development of other cancer therapeutics. May I have the first overhead, please?

First, there are too many possible reagents to be tested for the number of eligible cancer patients. Secondly, there is no consensus as to the most appropriate test for immunologic monitoring and to follow on the heels of that, there is little consensus as to even what biologic endpoints are acceptable in validating tumor vaccines. Could they be immunologic responses as intermediate markers or tumor responses and when we talk about tumor responses, we ask what kind.

There is also, I think, too much religious zeal based on preclinical belief systems. All these I'll be addressing.

Addressing the first point, the availability of vaccine reagents are absolutely staggering, at least to somebody who has been working in signal transduction as it relates to chemotherapeutic agents. It seems that many molecules, properly processed and presented can be a tumor antigen and that in vitro modifications of an antigenic peptide can render that peptide even more immunogenic, just MART 1, GP100, tyrosinase, P53, RASP, BCR able, MUC1, PSA, CEA and HER 2 are just a few agents that are already in clinical trials.

Moreover, the synthesis of immunizing reagents from peptides to naked DNA is relatively easy as compared to the chemical synthesis of chemotherapeutic agents. This encourages the production of a large variety, not necessarily a large quantity, but a large variety of vaccine reagents and this problem is further compounded by the proliferation of immuno adjuvants such as GMCSF and IL-2 and of co-stimulatory molecules. The commonatorial possibilities of these clinical reagents are clearly unbelievable.

Another issue is the restricted nature of how antigens bind to specific MXC molecules. In effect, this significantly reduces the number of eligible patients for any vaccine trials since HLA-1, HLA-2 or HLA-3 all may have different epitopes that it presents.

This number is further reduced by the fact that only those cancer patients with intact immune systems are expected to achieve maximum benefit from tumor vaccines. This last belief has prevented the application of hard-nosed go, no go decisions commonly used in the prioritization of chemotherapeutic agents. Vaccines that give no therapeutic responses are often excused by the

statement if we only tested our immunogen on patients with earlier cancers.

Grouping the second and third points, I have found it peculiar that despite the vast knowledge base in immunology, scientists cannot agree on the acceptable endpoints for the clinical validation of tumor vaccines. Should there be tumor shrinkage? Increase in timed progression? Reduction in relapse and adjuvant treatment trials or simply a laboratory immunologic response such as generating a cytotoxic

T-cell response or increase in precursor frequency?

Whereas most, but not all, would agree that the gold standards could be tumor shrinkage and/or the generation of cytotoxic t-cells specific to autologous tumor, certain realities may preclude the use of these parameters.

For example, the inability to access and to grow autologous tumor cells severely limits this monitoring approach in common cancers such as prostate cancer. This then mandates the availability of intermediate immunologic endpoints and as mentioned before, unfortunately there doesn't appear to be a consensus of which endpoints are

acceptable as an indication of a positive laboratory response.

Peculiarly, very few academic investigators would be willing to kill a specific vaccine program based on poor performance as measured even by the gold standards, possibly because there are just too many theoretical plausible excuses for a nonresponse. For example, and I think all of you have either heard or given these excuses --

(Laugher.)

-- the patients are too immunologically compromised. We just don't know what the right laboratory test is. The wrong cytokine was used as an immuno adjuvant. There is a better form yet to come of the failed antigen NMI pipeline.

This then leads us to the third point and one that is both amusing and troubling as a nonimmunologist observer and that is how much religious fervor is invested in the specific vaccine approaches? My friend and colleague Matt Cheaver stated beautifully in a recent talk when he compared the current debates in clinical tumor vaccine field in theologic terms. He observed that tumor immunologists are all of one religion, centered on

the belief that tumor vaccines will work. But that this religion is broken up to many sects, all proclaiming to be true believers. These sects arise from students of influential mentors and from experience with specific experimental systems, that experienced scientists who conduct their animal and in vitro studies with iron clad rigor would not only accept, but champion results from substandard clinical investigators is sociologically amusing.

However, that we spin wheels by not setting firm endpoints for go, no go decisions in clinical trials because of our personal belief systems only is I think scientifically unacceptable.

These sound like serious problems for clinical tumor vaccine development. However, I'm actually very, very sanguine about the future of your field, partly because I'm not in it.

(Laughter.)

In actual fact, this is because from my vantage point the critical issues that I've just outlined to you facing the field are not fundamental scientific roadblocks. The basic building blocks of your theology are real. But organizational problems relating to standards and priorities persist. The standards and priorities are set by consensus which

basically means that the vaccine community has really the solutions at hand. You just have to get together, spend time, discuss and have discipline.

You might want to consider through workshops like this the development of consensus of what laboratory tests may direct the developmental stream and I know that that is the goal of this workshop.

Details should include which laboratory parameters against what gold standards and how these labs are performed. You might also consider developing an immunologic competence grading system akin to the Karnofsky status that we use in cancer, or the CD-4 counts used in AIDS treatment, so that clinical vaccine trials can be rationally compared and the condition of the patients on entry standardized.

Next overhead, please. Lastly, you should seriously consider creative and clearly out of the box approaches to clinical trials design that may overcome some of the problems stemming from too many reagents and too few patients.

It is possible to test -- it is impossible to test all the theoretically pertinent combinations of immunogen adjuvant disease state and

immunizing regimen especially in Phase 3 studies. Instead, we need expedited approaches to interrogate the efficacy of specific combinations.

Given that most vaccines have low toxicity, should we not limit the goal of a Phase 1 vaccine study to be simply dose finding, using a laboratory endpoint within plausible ranges? Novel design approaches such as randomized Phase 2 trials and I've listed some of the articles that you might want to read permitting less stringent false positive rates that is using P value, acceptable P values of .1 or .2 and not relying on .05 and more stringent false negative rates, a beta of .01 may be in order.

Factorial design to accommodate combinations in a most parsimonious way and sequenced randomized Phase 2 studies will permit the use of fewer patients per treatment arm.

Obviously, some of these approaches should be used only in the initial exploration of optimal combinations with the testing of optimized regimens and standard Phase 3 studies. And certainly, you'll want to make sure that the FDA and other regulatory agencies buy into this concept. But using these design approaches in some cases, at

least theoretically as outlined in these articles, reduce the patient requirements by many fold.

Dr. Richard Simon who actually pioneered many of these concepts who I believe will be a member of the workshop and I suggest that you talk to him about some of these ideas.

Earlier on I made the disclaimer that I am not an immunologist, as an honest appeal to be gentle on my commentary. However, I also know that such a disclaimer did not help Richard Nixon when he said, "I am not a crook."

(Laughter.)

So will gratefully accept any criticism as an opportunity to learn and learn for my division who does a lot of clinical trials and vaccines.

Deep down inside, however, I am very jealous of you for working in a field that is likely to produce a nontoxic therapy where the clinical investigations are steeped in mechanism based science and where there are so many challenges that can be readily overcome.

Thank you very much. I hope you have a good conference.

(Applause.)

DR. PURI: Thank you very much, Dr. Liu. I think Dr. Liu has set the stage of the major goals of this workshop and we hope that we'll be able to achieve some of those in two days of workshop.

Before I introduce our next speaker for plenary talk, I would like to pass along a few additional housekeeping information for you. Please note that we will have a poster session scheduled for viewing today beginning at 5:30 p.m. to 8 p.m. This session will be held in the same area where the refreshment breaks are held. There are 18 posters. Some of them are invited posters.

I would like to thank poster presenters for your participation and sharing your data with all of us, particularly in a very, very short notice. All poster presenters are requested to be present at their posters from 5:30 p.m. to 8 p.m. today, although the posters will be left in place for additional viewing tomorrow until 3 p.m., but poster presenters are not required to be there except for today.

Please note that lunch will be on your own each day. The first floor cafeteria on the basement level and the second floor cafeteria will be open. There are additional cafeterias in the

campus, building 31, building 45 and building 1. They are located about 5 minutes walking distance from here. It may take slightly longer because there is a lot of construction going on around the campus.

The map of the campus is provided at the Registration Desk. Please look for these at the Registration Desk. If you should need further assistance throughout the workshop, please feel free to speak to anyone at the Registration Desk or with me.

Now it gives me great pleasure to introduce our speaker for the plenary talk today, Dr. Ralph Steinman. Dr. Steinman is a professor and senior physician in the Laboratory of Cellular Physiology and Immunology and Henry G. Kunkel Professor at the Rockefeller University. After obtaining his medical degree from Harvard University and medical training at Mass. General Hospital and research training at Rockefeller University, Dr. Steinman has held various positions at the Rockefeller University. Dr. Steinman is an editor and advisory editor of several distinguished scientific journals including editor of Journal of Experimental Medicine. He's a member of many

national and international societies and scientific advisory groups of many national and international panels and institutes.

Dr. Steinman has served as a chairperson of several keystone symposia including one on the dendritic cells. Dr. Steinman has given numerous honorary lectures and received various distinguished national and international awards.

This year alone, Dr. Steinman has received Foley medal from Cancer Research Institute, honorary doctorate from University of Innsbruck, Austria and Max Planck award from Alexander von Hamble Foundation.

Dr. Steinman has done pioneering work on dendritic cells. As we know, that it is DC that has generated a new tremendous interest of immunologists. The review article by Jacques Banchereau was also a speaker and present in the audience today. And Dr. Steinman published in Nature this year and it has become an important article referring to dendritic cells.

With this note I would like to invite Dr. Steinman to tell us everything about dendritic cells and the antigen presentation.

Dr. Steinman, please.

DR. STEINMAN: Thank you very much, Raj. Thank you for bringing so many right people to the right place at the right time.

You know the joke, ladies and gentlemen, about the role of plenary speakers at meetings. It's a bit like a corpse at a funeral. You have to have one, but you don't expect them to say very much.

(Laughter.)

I'll try to overcome that reputation, but who knows.

I'd like to actually discuss three topics that we're going to be considering in the next two days. One are some results, the first results in our lab on the active immunization of humans, particularly volunteers, normal, healthy adults with dendritic cells. This work has been led by Nina Bhardwaj and Madhav Dhodapkar at the Rockefeller and we're working closely with Gerald Schuler in Erlangen in Germany.

Then I want to look at the processing of cellular derived antigens by dendritic cells. There are many different forms of cells that can be handled by dendritic cells. I'll be going over the work at Kayo Inaba and I have been doing in the

mouse and I'll refer also to the work of Nina Bhardwaj, Birthe Sauter and Matthew Albert in the human.

And the third topic I want to think about with you is the manipulation of dendritic cells in vivo. I know this conference is dedicated to the use of ex vivo manipulated dendritic cells, but ultimately we really want to go to the dendritic cell as it exists in situ and we certainly have to keep in mind their physiologic features as we design ex vivo studies.

Now before getting into these three topics I thought I'd just remind us of a few things. The first is that -- is diagrammed here. Immunologists often are able to identify antigens, clinically significant antigens, but we lack adjuvants, to use those adjuvants to properly control the immune system.

Melanoma is certainly the best example in human cancer. We have lots of candidate antigens and we need to know how to use these antigens to elicit strong T-cell mediated immunity with the assumption that if we were to do that, we'd have a significant anti-tumor effect.

HIV is the classic example now of a chronic viral infection where we've known of several antigens for so many years and yet we still don't have a candidate vaccine. That means we still don't have a way of actively immunizing individuals to HIV-1. And then there's the reciprocal thing in autoimmunity, particularly a disease like insulin dependent diabetes where we're going to be identifying auto antigens and we're not going to know how to use those antigens to turn the immune system off.

So the potential role of the dendritic cell system is to give us a new way of using particular antigens or cells to manipulate the immune system.

The reason they're so attractive at this stage, I guess can be boiled down to these three points. One is that they're very potent, that is, relatively small numbers of cells, relatively small amounts of antigen induce strong T-cell mediated immunity. And all kinds of T-cells immunity, depending on the type of antigen and the type of stimulus that's delivered with the dendritic cell.

They prime T-cells. They initiate immunity both in vitro and in vivo and they do this

without any other adjuvant. They are nature's adjuvant.

And then finally is that this is the physiologic system that can be used to manipulate the immune response. And let's just go over some of these features of their physiology. They're located at body surfaces. The best studied are the skin and the lung and what you're looking at here in brown are the dendritic cells in the rat airway epithelium. This is a micrograph that was given to me by Patrick Holt. And you see that relative to the number of the nuclei stained in blue, the number of round dendritic cells isn't very great. The actual number in there, the epithelium isn't known, but it's relatively small. But their size and their distribution is just right to pick up antigens that are entering into the body.

And then, of course, they're very abundant in the T-cell areas of the lymph node tissue. This is a low power of a mouse lymph node. The blue is a
T-cell marker, CD4 and the brown is the MAC Class 2 type of antigen presenting molecule. And what you see is that the T-cell area is just loaded with

these stellate intensively positive MAC 2 expressing cells.

And again, there's only one or two percent dendritic cells in an entire lymph node suspension, but you can see that their size and their distribution is just right for manipulating the T-cell component of the immune response.

This is just a little cartoon summarizing the distribution of dendritic cells in vivo at body surfaces in the interstitial spaces of many organs such as the dermis. In the circulation, particularly the afferent lympathatics and in the lymph node tissues, particularly, but not exclusively in the T-cell areas.

And again, it's known that if one were to administer an antigen into the skin and isolate these veiled cells as they were first called in the lymph they would be carrying the antigen in a form that's highly immunogenic for T-cells. Or also if one injected an antigen into the skin and isolated the dendritic cells from the lymphoid tissue they too would be the main site in which immunogenic antigen is available for presentation into T-cells.

And then there are the experiments as we'll go over in a moment where one takes dendritic

cells themselves and pulses them with antigen and you can observe them in experimental animals, homing to the T-cell area and initiating the immune response there.

Now there are many components to this physiology in vivo. I've just broken down some of them. Their mobilization, that is, how do you mobilize dendritic cells from tissues that are already there and how do you increase their numbers? Their maturation, this is a complex process that I'm going to come to next that greatly influences many of their functions. Their migration, how do they know to go to the right place at the right time and then something that I'm going to end on in my talk is their short life span once they're fully mature, their mortality and I'm going to emphasize that even though we're injecting dendritic cells in relatively small numbers, in turn, a very small fraction of these actually make it in a live state to the T-cell area. So we're working now at a very suboptimal range in terms of the efficacy of the dendritic cells we inject.

So a little more on this topic of maturation before I get into these three topics, it's proper that I introduce before. This is a

concept that was first described by Gerald Schuler in studies of Langerhans cells. But it's come up again and again with dendritic cells that have been isolated from various tissues such as the rat lung, human blood, mouse spleen, the various bone marrow cultures that we're using to generate dendritic cells and even the blood monocyte system that's very popular now. And basically the dendritic cell has two phases of function, one called immature in which it's actively taking up antigens and another called mature where it's a very potent T-cell stimulator. I think the term mature, it's just a word, is appropriate because this is the end stage of dendritic cell development. It's often very short-lived, living only a day or two and it's fully functional in terms of its characteristic function that is potent T-cell stimulation.

And it's a cell that we can't convert into any other cell type so it seems to be the terminal stage of differentiation.

Now in terms of antigen uptake there are a number of ways that these immature dendritic cells can capture antigens. Phagocytosis is one of them and we'll be discussing the uptake of apoptotic and necrotic cells. They can take up pinocytosis --

they can take things up just by fluid phase pinocytosis and lucifer yellow is a convenient experimental market, but this is the way dendritic cells are handling standard soluble proteins which are really relatively an efficient way of delivering antigen and often given in doses of hundreds of micrograms, even milligrams per ml.

And then there are the more interesting pathways of absorptive uptake and I've only listed a couple of them, but this really does look like an expanding area in many ways whereby dendritic cells may selectively bind things. And this may be very, very important in terms of targeting antigens to the immature stage of dendritic cell development.

Maturation is induced by a number of things, lipo-polysaccharide being a typical one in the lab, living bacteria, certain viruses like influenza and then a number of inflammatory molecules such as IL-1 and members the TNF family. The TOL family has been implicated because of a molecule called RP 105 which is very TOL-like in its structure and antibodies to RP 105 can stimulate dendritic cell maturation.

Now this matured dendritic cell is really exquisitely differentiated to initiate

immunity. They have a number of adhesion and co-stimulator molecules. These are just a few. They resist the immuno suppressive effects of IL-10. They express a repertoire of chemokine receptors that's quite selective and what's interesting is that the corresponding chemokines are expressed constituentively in the T-cell area it seems.
And then finally they have very high levels of MAC peptide when this being measured directly.

I just illustrate some of these features of dendritic cell maturation. This is the prototype Langerhans cell and it's stained for MAC Class 2 in green and a lysosomal marker in red. This is the mature Langerhans cell driven just simply by placing skin cells in culture and what you see is that most of the MAC 2 molecules are on the surface in green and that there are relatively few lysosomes.

Now this cell is a very potent stimulater on the mixed leucocyte reactions and mitogen responses and super antigen responses, but it doesn't capture any soluble antigens. It stopped endocytosing. It's put out all its MAC onto the cell surface it seemed. But it derives from the resident immature cell in the skin where it's just the reciprocal occurs. That is, the MAC 2 is

sitting within the vacuolar system in the right place to accept MAC peptide. And in fact, some of the new monoclonal antibodies that are being developed to look at MAC peptide complex formation such as the very lovely reagent that's come out of Ron Germaine's lab called C4H3. It's a peptide from heneg lysosome presented on mouse MAC 2. You can follow the formation of MAC peptide complexes in dendritic cells. It begins within these MAC 2 compartments and then moves to the cell surface and is very abundant there.

So the immature dendritic cell is designed to capture and make MAC peptide complexes and then the mature dendritic cell is the one that presents these to the T-cell system.

And just to look at the expression of co-stimulatory molecules this happens to be from the mouse. A few of them are listed on the X axis here, CD 86, CD 54, CD 40. And what we're looking at are immature dendritic cells from mouse bone marrow cultures and then in parallel dendritic cells that on the last day of culture received a stimulus of an nanogram from MLPS.

What you see is that the maturation stimulus greatly increases the level of CD 86, CD

54, CD 40 and these are the mean florescent index of all the profiles in the culture. So this again is really just the right cell to stimulate the T-cell.

Now one last introductory slide and that is the rationale for using dendritic cells as nature's adjuvant has come out of many, many experiments in mice which were designed as shown here. Dendritic cells are removed from one animal, either from the lymphoid organs or they're grown up from progenitors, particularly the bone marrow. And then they're charged with antigen ex vivo and then they're

re-infused into syngeneic animals. And what one observes, initially, was the induction of CD 4 immunity, but several of the people in this audience have pursued this in the context of CD 8 T-cell immunity.

And then there have been much prettier assays of immunogenicity, that is the induction of protection against tumors, protection against viral and bacterial infection and also the elicitation of autoimmunity when there's an appropriate auto antigen being delivered.

Now what was shown in these experimental systems was that the dendritic cell that one

injected was really controlling the immune response so by using MAC restriction F-1 animals were immunized and if one used parental strain dendritic cells only those T-cells that saw the MAC of the dendritic cell that you injected, only those T-cells were immunized. So the dendritic cell is not just schlepping in antigen for the host to present, it's directly stimulating the recipient. And it's not surprising therefore that one typically needs live dendritic cells to see this immunogenicity.

So now let's get on to human studies. Now there are many ways that are being used to make the dendritic cells. These are diagram of the two most popular ones, I think. One is to grow them from progenitors in the CD 34 fraction and the two key cytokines are GM-CSF and TNF although other cytokines may be used to maintain the progenitors such as CK ligand and flt3L. And what happens in these cultures is that the dendritic cells grow up in these distinctive aggregates and if one looks as the aggregates, they often have their MAC 2 within these intracellular compartments. So this would seem like the right stage to be offering them antigens.

I just want to stress the very lovely work that came from Christophe Coe and Jacques Bancherau that there may be two types of -- there are two types of dendritic cells being generated in these cultures, one with the features of Langerhans cells and the other which are called monocyte-derived dendritic cells or dermal-type dendritic cells. And we really don't know a lot yet about the relative immunizing function of these two types of cells. The one functional difference that's known, for example, is that at least in a tissue culture system these monocyte-derived dendritic cells have a capacity to stimulate B-cells as well as T-cells and the Langerhans cells lack this capacity.

Now the other system that's being used is the blood monocyte and this is being expanded into a typical matured dendritic cell. This is the one that we're using in our studies. It's an accessible population. It's the most homogenous preparation of dendritic cells that we can make in the lab and it's also the most potent. And what's done is that the precursor population is cultured in GM-CSF and IL-4 for several days. We are just using plastic adherent cells currently as the precursor

population so it's enriched in monocytes, but hardly pure.

And then you get a cell that has many of the features of an immature dendritic cell. It's not exactly like the Langerhans cell, but it's not fully differentiated and to do that we had a maturation stimulus and for clinical studies we use just a condition medium that's derived from monocytes applied to immune complexes. And then you get the mature dendritic cell. And what's diagrammed here in black is that that's the cell that has very abundant MAC 2 on the surface as well as membrane co-stimulatory molecules and just a few lysosomes inside the cell in red.

And then there's some markers whose function that we don't know, but they're very useful for monitoring the maturation process and three of them are surface expression of CD-83, member of interglobulin super family, p55 which is a cytoplasmic protein of unknown function, thought to be an actin bundling protein by sequence somology and then a new marker that's found within the lysosomal system called DC-LAMP and it was identified in DARDA at the time Jacques Banchereau was directing that institute. It was recently

described in a paper in Immunity by Serge Le Bec, et al.

As I understand it, this marker which was kindly provided to us by Drs. Le Bec and Sam Salen, this will be available from Immunotech. It's an intercellular antigen and the lysosomes of these mature dendritic cells and it's quite a lovely marker.

Just one word about the heterogeneity of dendritic cells. Heterogeneity is just a word. We really need to define it in clear terms and not be frustrated by it. There are many reasons for it. Stage of maturation is one of them. I've mentioned the possible differences between Langerhans cell type versus monocyte-derived dendritic cells and finally, there's this one that's often called myeloid v. lymphoid or, by Jung Jin Lu, DC-1 versus DC-2.

This distinction is the one we have the least handle on right now, but it could be very important. The idea is although there's still not a lot of data, is that the lymphoid cell is more specialized for immune regulation and possibly deletional tolerance rather than immune activation. So we still don't have our hands on buckets of

lymphoid-type dendritic cells, but this may be an important thing for the future.

Okay, so here's what our cells look like. They're large, stellate, actively motile cells and we've selected the mature stage for our studies because the mature dendritic cell is the one that presents peptides very efficiently to CD8 positive autologous T-cells.

Now the point is that when these cells are cultured in the absence of the cytokines in which you reared them, they maintain this shape and their -- all the differentiated functions that I outlined. And that's very important. In other words, they appear to be terminally differentiated.

This is just a look at some of their features. MLR stimulation, even though it seems like a relatively crude assay is really a very reliable one. These mature dendritic cells at very low doses, less than one dendritic cell per thousand allogenic T-cells stimulate very strong MLRs, easily greater than 50,000 counts, measured at Day 5 of the MLR, not the usual Day 7. So they're very potent stimulators and really all the patients that we have studied give you dendritic cells that stimulate MLRs like this. We have a standard T-cell donor as our

allo, but frankly, almost all allogeneic T-cells will give you the same results.

This is just the phenotype of the cells and I was a little upset, but these have been gated for the large cells and we have small cell contamination because we're using adherent cells as the starting population and the frequency of small cells in our preps is anywhere from 15 to 75 percent. But the large cells reliably have this phenotype. The background is less than a log on the facts. CD83 staining is very strong on most of the cells, generally over 95 percent of the cells and the levels of HLA-DR and CD86 are very high. This is a directed immunolabel.

And then CD14 which was present initially at high levels on all he monocytes can't be detected either on the cell surface or intracellularly.

Okay, so now for the study. We've injected about 2 million mature monocyte derived dendritic cells subcutaneously in the upper arm. They've been pulsed with KLA as a priming protein, tetanus toxoid as a boosting protein or the influenza matrix peptide that's immunodominant on HLA2.1.

And then controls, as I'll go over in a moment are to first inject the patient with dendritic cells that have not been pulsed with antigen or we have given just antigen in the absence of dendritic cells to four individuals.

We pulse the antigens on during the maturation phase, that final two days of culture in the monocyte condition medium.

And then this is just the little schema of when we measure immune responses and the various phases of the study. First, there are base line studies where we measure immune responsiveness by the various parameters that I'll go over, at least two and usually four times over a several month interval.

Then we give the control dendritic cells, that is, those that don't have any antigen on them and we measure the immune parameters again at Day 7 and Day 30 and then we gave the antigen pulsed dendritic cells and looked at the responses at Day 7, 30 and beyond. And we've just done a single primary injection so far in nine healthy adults.

So this is what all the volunteers did in response to KLA. We're measuring the proliferative response to KLA just in PMBCs taken

from the volunteer. And you see we use 10 in 1 microgram per ml of antigen. We find at higher doses there is a proliferative response from the preparation we use without any priming of the individual. So you see here are several base line pre-injection values are relatively low following the control dendritic cells. There's no change and then when you give the KLA pulsed dendritic cells, you see a nice boost in the proliferative response, including to a very low dose of antigen a microgram per ml.

And then these are proliferating cells, are CD4 positive because if you deplete CD4 positive cells prior to assay you lose the response.

And so this is the summary data for all nine individuals, D1 through 9, measured pre-injection following the injection of control dendritic cells or following the injection of KLA pulsed dendritic cells. And you see that all nine individuals show a nice injunction of KLA specific -- KLA dependent proliferation.

The four individuals who received KLA only. This was a dose of 4 micrograms. Were not primed. And the dose of 4 micrograms was chosen because that would be the dose of KLAs that would be

present in the inoculant if we had not washed the cells at all prior to injection.

Now we've also looked at the CD8 response by virtue of having the influence of matrix peptide on these dendritic cells and many of our donors were HLA2.1 positive so they could respond to the matrix peptide and several were HLA2.1 negative so that they shouldn't respond to the matrix peptide.

And what we did were LE spot assays for gamma interferon producing cells and these LE spot assays were done on PBMCs taken from the patient and this is just an overnight culture. So there's no expansion of the PBMCs in culture for a long period as if often required to see CD8 immunity in humans. We're seeing this response directly out of the blood. And the way these assays are set up we always have a control peptide. This is the gag peptide from HIV that's dominant for HLA2.1 and the matrix peptide. And what you see is that in all the individuals that we injected who were 2.1 positive, there's a boost in the gamma interferon secreting cells. These are all CD8 positive cells rather than CD4 positive cells.

And it's antigen specific, that is, you see a good matrix peptide, but not with gag peptide. The 2.1 negative individuals didn't respond and the people who got 4 micrograms of peptide subcutaneously didn't respond.

And then this just shows you another assay that is the capacity to generate CD8 positive killer cells that are specific for the viral peptide or an influenza infected target. And what we're doing is we're measuring the matrix specific CTL response in a chromium release assay in T-cells that are boosted with autologous dendritic cells for 7 days in culture. And controls are done with and without antigen. These are the responses with antigen. And what you see is that following the injection of control dendritic cells, that is, without matrix peptide, there is very low levels of lysis, but then when they get the MP pulsed, peptide pulsed dendritic cells, you get very nice boosts in their responsiveness as measured by a CTL assay.

And furthermore, you can now detect a specific HLA2.1 MP peptide tetramer binding in these recall responses in vitro. So we're definitely priming the CD8 compartment with the single dose of

monocyte derived dendritic cells given subcutaneously.

So this is just a little summary of the way many of these ex vivo studies are being planned. One first has to get a hold of dendritic cells and generally these are being generated from precursors rather than being taken fresh out of the donor blood.

And these are grown up in various ways. We're currently emphasizing the monocyte derived pathway. You then have to charge them with antigens of therapeutic importance and then you reinfuse them and have to measure the T-cell response.

Now there's a term that's coming up called translational research with this kind of experiment. I hate this word. It is not translational research. Just because it was done in a mouse doesn't mean it was just a bunch of translation to human. This is research. Every bit of this is really serious research and there's an awful lot to do. We have to learn how to make the different dendritic cells in the different subsets and test them. We have to come up with ways to deliver antigens and to measure the efficacy of that antigen delivery to the dendritic cells and we have

to learn how to measure the human T-cell response which is not exactly easy. It's not been that easy to do that and many of the newer assays such as LE spots and MAC tetramer binding are really just wonderful in this regard. We're going to have to see how they behave and what they mean.

Okay, on to the second topic and that is the delivery of antigens to dendritic cells. I'm looking around for a clock. There's not one in this room that I can see. I think a very important breakthrough was made in my colleague Nina Bhardwaj's lab, particularly by a really brilliant M.D. Ph.D. student, Matthew Albert and we were all working with Birthe Sauter who is a dermatologist from Erlangen. And what they were looking at was the response to influenza in HLA2.1 positive individuals in tissue culture. It was known from Nina's work that dendritic cell and HLA2.1 individuals and in other individuals were very potent APCs for CDA positive CTL response.

So what they did was they put the source of the influenza peptide in an HLA2.1 negative cell and that cell was either infected with influenza or it was transfected with the influenza matrix gene. What they found is that if they made these A2.1

negative cells undergo apoptosis that the dendritic cells would take them up and present them on Class 1 to autologous CD8 cells. And a subsequent study with Bob Darnell has shown that a candidate tumor antigen in perinea plastic disease, cerebellar degeneration antigen called CDR is also presented in this system in this way.

Now they had to use the immature stage of dendritic cell development to get good presentation and it turns out that the immature stage has a vitronetrin receptor, alpha V beta 5 that seems to be involved in the uptake of apoptotic cells.

And what's fascinating about this system is that the dendritic cells present antigens on Class 1 whereas if you use monocytes as the APC, they do not present the antigen on Class 2, even in rapid chromium release assays. So there seems to be a very fundamental difference between the dendritic cells that can present antigens through this exogenous pathway and the macrophage which scavenges and destroys antigen through this pathway.

Now my colleague Wanaba and I have been looking at this using the mouse system because there we have antibodies to MAC peptide complexes. So you

can put the source of the peptide in one cell and the MAC on the dendritic cell and then start monitoring this capture of peptide from cellular antigens rather directly.

And the monoclonal we used was the first monoclonal of this kind that sees an MHC peptide complex. It's called a Y-Ae because the presenting molecule is an I-E molecule and the peptide is derived from the I-E product, another MAC molecule by chance. The beauty of this system is that this MAC peptide complex is expressed at very high levels on dendritic cells in mice that carry both IE-alpha and the I-Ab genes.

So what we did was simply put the peptide in one cell which, of course, was a B cell since that expresses a lot of I-E and then we used the dendritic cell from I-Ab of mice. Now we found that the dendritic cells, in order to capture the peptides from the B cells had to be at the immature stage of development and in addition they had to receive a maturation stimulus for us to see very nice MAC peptide complex formation.

So this is what it looks like. You can barely see on the Y axis is the staining with the Y-Ae monoclonal antibody and all the staining is

specific. It's not seen with the isotype control and on the X axis is a staining for a maturation marker, in this case, CD86.

So if you offer these cells the preprocess peptide you see a nice signal developing on these dendritic cells that have matured during a one day culture with the peptide or with the dying cell. And then this is the signal where you give them B-blasts and these are apoptotic B-blasts and these are B-blasts that are killed with antibody in complement. Now both apoptotic and necrotic cells are processed very efficiently in this system whereas in the Class 1 presentation system that Nina Bhardwaj and her colleagues have studied, only apoptotic cells are presented through the exogenous pathway. And then here you see what happens if you separate the B-blasts from the dendritic cell in a transwell. There's very little formation of MAC peptide and here's what happens if you add the B-blast to the already matured dendritic cell. It no longer captures these cells to make the MAC peptide complex.

And this is what the cells look like -- studied by confocal microscopy. These are studies that we've been doing with Ira Melman's lab at Yale

and in particular with a wonderful graduate student named Shannon Turley. So this is a single immature dendritic cell stained for MAC 2 in green. So you see these MAC 2 rich intercellular compartments and in red is a marker for the B cell that we're feeding to the dendritic cell and you see the immature cell as just taking up a lot of B cell fragments and in the overlay you see that many of the B cell fragments have localized to the MAC 2 compartments.

Now I must say this is not a typical example. Most of the immature dendritic cells only have a few fragments, but we've just done this, taken this example to emphasize the targeting of the B cell to the MAC2 compartment.

Now what one can do is isolate these cells, sort of block this uptake phase by culturing, doing the whole culture in the presence of ammonium chloride which blocks processing of these phagocytosed fragments. So you can then purify the dendritic cells, remove the ammonium chloride block and show that the cells then rapidly form MAC peptide complex on the surface.

The efficiency with which dendritic cells can make MAC peptides from other cells is really impressive, we feel. We've quantitated --

tended to quantitate the level of IE protein that's in the B cells that we offer to the culture and the total amount of protein is about .3 nanomolar and most of that protein is actually going to the macrophages that are eating more actively than the dendritic cells in the culture.

And this is the level of preprocessed peptide one micromolar that gives a comparable signal to that level of protein. So it seems that delivering the protein in a dead cell, a dying cell is a much more efficient way than giving peptides. And as you know, peptides have been used for a long time to try to manipulate the immune system of mammals and they're not very effective, not very efficient. It may be that by learning to target dying cells to dendritic cells we're going to make a huge leap of efficacy.

Now to some extent this efficacy is due to the efficiency of phagocytosis. That's an absorptive uptake mechanism, but we showed many years ago that that gives you roughly a thousand to three thousand fold enhancement over fluid phase. But the trouble is that just gets the protein into the cell, so the dendritic cell must be very efficient at converting that protein to the MAC

peptide complex relative to delivering the preprocessed peptide to the very same cells.

So just to sum up some of the antigen handling properties of dendritic cells, they seem to have a number of receptors that are going to be very interesting to study as delivery system and possibly the vitronetrin receptor is going to be very important for apoptotic cells because that's the one that's expressed selectively on immature cells and antibodies to it block uptake of apoptotic cells. They express very high levels of MAC peptide and what may be qualitatively very special about a dendritic cell is this exogenous pathway. It's been found not only in apoptotic cells, but also by Sebastian Emovengrena with immune complexes. So it can be engineered so it takes up immune complexes, but it won't present on Class 1 and the dendritic cell is very efficient in that regard.

The M2C compartments are of great interest and it's becoming clear that their function is regulated very beautifully in the dendritic cell, so for example, in the maturation stimulus is given, this inhibitor, cystatin C disappears from the M2C and that allows cathepsin S the cystine proteus as blockbust the cystatin C to be more active and that

degrades the envarian chain and for one thing allows the MAC peptide to move to the south surface.

Finally, there are markers like DC LAMP whose function we really don't know yet.

Okay, now for the last topic, that is the mobilization and manipulation of dendritic cells in vivo. This is one way of looking at this pretty substantial question and that is to think of the dendritic cell as being of several types of proliferating progenitor, a precursor such as the monocyte that doesn't really have any features of dendritic cells, but can become one. Cells that are now starting to look like dendritic cells, the immature ones, but still need a maturation stimulus to be the potent stable T-cell stimulator. And then finally this last stage wherein the dendritic cells undergo apoptosis. And I think all these stages must be kept in mind as we work with these cells and as we try to devise ways of manipulating them in vivo.

There's already a lot that's known. For example, flt3L seems to be a very active stimulus for mobilizing various precursors in immature forms of the dendritic cell from the proliferating progenitor. There's evidence now that among site

can start developing into a dendritic cell when it moves across an endothelium and also receives a phagocytic stimulus. These are studies of Gwen Randolph that was recently published in Science.

The multi-drug resistance receptor, oddly enough, may be involved in the mobilization of Langerhans cells from the skin, drugs like Reserpine and Verapomil are very effective at blocking the immigration of Langerhans cells from the organ explants of human skin.

Then there are the members of the TNF family, that for one thing prolong the viability of dendritic cells. And then they're going to be select chemokines that influence the targeting and movement of dendritic cells in vivo. I just want to finish with this last point. That is, when we inject dendritic cells, at least these mature and immature ones, they're very rapidly undergoing apoptosis in vivo. So we're really losing a lot of their efficacy.

So the way this experiment was done is one injects dendritic cells into a mouse and these can be syngeneic or I want to show you what happens in the allogeneic system because what happens is that when the dendritic cells are injected, they're

actually processed by the recipient's dendritic cells. So we take dendritic cells from an I-E bearing mouse and we inject it into a C57 Black 6 mouse and then we look for the development of the Y-Ae epitope on the recipient dendritic cells and what you see is massive development of the Y-Ae epitope. This is a high power view of the lymph node, the B cell follicles, the edge of it is in brown and the Y-Ae expressing cells are scattered about the T-cell area and if you look at this by fax, you see that when you inject an I-E bearing dendritic cell into the appropriate strain of mice, there's a nice development of MAC peptide complex on most of the dendritic cells marked by CD11C or marked by the recipient MAC 1-Ab.

Now if you look for the donor dendritic cells you see very few. You can do this in a syngeneic or allogeneic system. You can label the cells with a tracking dye or you can look for the I-E positive cells. So when we inject 500,000 dendritic cells, you're lucky to see a thousand Day 2. But yet all of the dendritic cells in the recipient seem to be expressing MAC peptide complex. So somehow they're capturing peptide from the live dendritic cell that we had injected.

So what we interpret is that the dendritic cells one injects in afferent lymph, they're known not to make it into efferent lymph, that they die very quickly upon reaching the lymph node. The T-cell may rescue them because those cells express CD-40 and the T-cells they active FCD40 ligin and that works to rescue dendritic cells in vitro.

But if they're not rescued, they're actually processed by the recipient cell and this is massive MAC peptide complex formation and we really do not know yet what the functional consequence of this is.

So again to summarize some of the things that may be going on when we're injecting dendritic cells in vivo and injecting different forms of cells in vivo, there are different ways that the dendritic cells can capture these and include capturing the dendritic cells themselves and setting into motion this very efficient processing on to MAC 2 and probably on to MAC 1.

So this is my final summary slide. It's a little dark. I'm sorry it's not showing well. But really to emphasize that there are two very different pathways of white cell differentiation,

one that leads to scavenger cells or macrophages and another that leads to very specialized antigen presenting dendritic cells. And it's very important to make these distinctions. If there's one thing that really must not be done is to refer to an entity called the macrophage/dendritic cell. There is no such thing. We wouldn't know about all these features about dendritic cells if they hadn't been separated from macrophages and most of what we know about dendritic cells are simply not seen in macrophages. So we really have to overcome this very traditional barrier in immunology and focus on these two different pathways of differentiation because they're very different.

The dendritic cell is dedicated to antigen presentation. It works in vivo, including in humans and it's very efficient. It's vacuolar system is very specialized to capture and process antigen. In fact, it's so devoted to antigen presentation that it seems to sacrifice itself to the process.

Thank you.

(Applause.)

DR. PURI: Thank you, Dr. Steinman. It was an excellent review of dendritic cells.

Everything we wanted to know. And very good data you have presented today.

Due to the paucity of time I think we have not have any questions at this time. Dr. Steinman will be here. He is chairing a session this afternoon as well. If you have questions, please feel free to ask him while he's here.

Time is 9:45 and it's time for our 15 minute coffee break. Please note as I indicated before that complimentary refreshments are provided and the set up is located in the main area lobby of building 10. For invited speakers there is coffee in the back stage so please stay here and have coffee in the back. Thank you very much. We shall return in 15 minutes.

(Off the record.)

DR. KEEGAN: Thank you, Raj. I'm Dr. Patricia Keegan with the Division of Clinical Trial Design and Analysis in the Office of Therapeutics and in contrast to what you received in your packages, we've slightly changed the order of presentation. We thought it might fit better to provide an overview of the clinical development process first as a lead in to several of the next speakers and so the first presentation will actually

be our discussion of the clinical development process.

In this presentation what I'm attempting to do is first to provide an overview of the development process for anti-neoplastic agents which is traditional cytotoxic chemotherapy as a background. I'm sorry for those clinicians in the audience for whom this is rather basic material, but because the audience is rather diverse we wanted to provide a general overview in this regard to discuss a little bit the areas in which FDA feels that they are important to have interactions with drug developers throughout that clinical development process and what the goals and purpose of these interactions are and finally to provide some thoughts on special considerations in the area of tumor vaccine where there are distinctions or differences in the approach to the development process for this product class which I would like to highlight or contrast as relative to the traditional approach for cytotoxic chemotherapeutic agents.

The clinical development process has been described as proceeding through a series of phases of drug development and all the oncologists in the audience will recognize the traditional Phase

1, 2 and 3 trial process. FDA also recognizes and has inscribed in its regulations a Phase 4 development process which occurs after product licensure.

In Phase 1 development, the purpose for cytotoxic agents is really a determination of a dose range which might be appropriate for future studies, and also to get some sense of the toxicity profile in human subjects. Because we expect that these agents will be toxic, these studies are generally conducted in patients with cancer where the risks are felt to be balanced against the potential benefits.

The considerations that one has to have in these trials is the number of patients to be exposed relative to the toxicity profile and to try and make this a judicious balance of the benefits and risks, that the monitoring for toxicity be appropriate relative to that expected toxicity which has been elucidated in preclinical animal testing. And in addition, that one should perform pharmacokinetic and pharmacodynamic analysis and data collection to assist in the development of the Phase 2 trials.

David, could you advance it? The next trial of development after an assessment of the dose range which is tolerable in human subjects is to address trials looking at an initial determination of the activity of the product in a fairly well defined and homogenous population. However, because Phase 1 studies are often done in very advanced patients, there may need to be further elucidation of an appropriate dose range. These again are studies conducted in patients with malignancy, although generally in patients with measurable tumor so that the activity assessments can be made. And again, considerations here are the number of patients to be exposed, generally ranging from 20 up to as many as a 100 with a focus on efficient determination of activity, again careful monitoring and characterization of the toxicity profile so that the monitoring process can be focused in future trials on those most relevant aspects of toxicity.

Next slide. The goals of the Phase 3 study then are to further characterize the safety and effectiveness of a product at this point in comparison to a control group. The standards of effectiveness vary depending upon what that control group is. One should need to look at equivalent or

superior activity relative to an efficacious or active control and clearly one would need to show that there is superior activity relative to an observational or an inactive or placebo type control. It bears, in fact, no standard therapy available for the disease being investigated.

The efficacy standards for Phase 3 trials are that the trials be adequate and well controlled. Well controlled would mean that the trials be balanced in the arms for relative prognostic variables and confounding factors which might confound the analysis of effectiveness. And there should, at the end of the trial, be evidence of a net clinical benefit or of an effect on a surrogate which is reasonably likely to predict clinical benefit.

The determination of net clinical benefit must be evaluated, however, in the setting of the currently available therapy for that disease and it must take into account the natural history of the disease or the clinical course following the standard therapy for a specific neoplastic subtype and stage of disease.

Potential endpoints which have been used in the development of anti-neoplastic agents have

most importantly looked at survival benefit, disease-free survival or time to treatment failure benefits, durable complete responses, durable overall responses, meaning complete and partial tumor responses or beneficial effects on disease related symptoms and/or quality of life.

Again, the endpoints need to be selected in consideration of what alternative therapy is available and what the goals -- what the potential benefits of those alternative therapies are.

Following the licensure or marketing registration of a drug, FDA notes that there can be additional studies to elucidate additional aspects of the product. Post-marketing studies further assess safety and/or efficacy information about a particular product and they may be required as a condition of an accelerated approval. Some examples of Phase 4 commitments might be to obtain information on late, sustained or delayed effects to determine their course, their severity, their incidents. One might further evaluate the pharmacokinetics safety and effectiveness in specific subpopulations and areas -- this has been a very active area of interest with the FDA to evaluate whether or not patients who are elderly,

the pediatric population, those with significant organ impairment may have a different safety and efficacy profile and often this is best characterized in later studies after the initial effectiveness from marketing has been obtained.

I want to speak a little bit about FDA interactions with clinical drug developers. The initial interaction with FDA are typically before the product has gone into any human subjects and maybe the so-called preinvestigational new drug development application stage. These conferences are held to reach agreement on the information sufficient to assure identity, purity and potency of the product which is a requirement for initial studies in humans often. It's necessary to reach agreement on appropriate preclinical studies to determine what might be a reasonable initial dose integration of therapy. There are some differences in the approaches that are used in classical anti-cytotoxic agents -- or in antineoplastic agents in the Center for Drugs where they have specified that acute toxicity data from two species involving one rodent and one nonrodent as well as histopathology results in one species be provided as that type of information. Because of the nature of the products

regulated in the Center for Biologics while this approach may, in fact, be appropriate for some drugs, one of the areas of focus is that the animal models, in fact, be relevant and the extent to which there is not -- the extent of information that's necessary in animal models may or may not have to be more heavily supplemented with in vitro data which will support the safety and the initial dose in the clinical studies.

In making a request for a pre-IND conference it simply requires a letter sent to the agency requesting that the conference take place and a listing of the proposed dates of availability. In an attempt to minimize the resources of the Agency so that we can focus on all aspects of trial development, we are now moving in the Center for Biologics towards teleconferences as the most efficient way to conduct these pre-IND conferences. At the time of the conference we will begin to -- at the time of the request we will begin to schedule a teleconference and subsequently we would expect a pre-IND package of materials to be provided as the basis of information upon which FDA will be able to provide some guidance.

This package should contain a description of the product, biochemical characterization to the extent possible and a description of the manufacturing process in brief. There should be relevant in vitro data. Where appropriate reprints of materials should be submitted. Animal toxicology and pharmacology studies should either be summarized or the proposals for such studies should be summarized and the clinical protocols should be outlined in sufficient detail so that one can make an assessment as to whether or not the information provided would support the proposed initial clinical study.

In addition, one should provide a specific agenda and the questions which need to be addressed during the course of that conference so that we can make efficient use of time. It would be anticipated that the Agency would review the package in its entirety prior to the conference and the majority of the time could be spent focusing on those specific questions.

And here's an address where the pre-IND materials would need to be sent. According to recent regulations these materials need to be received at least four weeks prior to the conference

call and the location of the address of the materials might vary depending upon the product. For products which are live vaccine, gene therapy products, which is not specifically the topic of this conference, the Office of Vaccines would be the primary office of review. For almost all other tumor vaccine materials it would be directed to the Office of Therapeutics Research and Review.

Once the IND has been submitted and clinical studies initiated, there are a series of additional meetings which FDA which schedule with the sponsor, a clinical drug developer to insure that there's efficient drug development to facilitate the drug review process and to provide input in that process. There is in the regulations a specification for an end of Phase 1 meeting under very specific conditions. That is, for products that would meet the designation of Subpart E and I will cover what that Subpart E designation is in a subsequent slide.

The purpose of an end of Phase 1 meeting would be concurrence that the drug, in fact, meets a Subpart E designation, review of the evidence of activity in Phase 1 and to reach agreements

regarding the design of a typical trial and standards for approval.

Next slide. That end of Phase 1 meeting is clearly the exception. Ordinarily, the Agency would not expect to meet with a sponsor until the end of a Phase 2 trial in which that initial evidence of activity has been determined. At the time of the end of Phase 2 meeting, we would review the results of the Phase 1 and 2 studies to determine and the evidence of clinical activity gleaned from those studies. We would attempt to obtain agreement on the design of Phase 3 or pivotal trials and we would again review the standards of approval in the drug setting, given the natural history of the disease and the alternative therapies available.

Following the completion of one or more pivotal trials, the Agency would then typically meet to discuss the results of those clinical trials. Again, the purpose would be to review the results of the trials and of the overall development plan and the entire body of evidence available which supports the safety and activity of the product and to discuss the future directions. For trials which appear not to have been successful, one might

discuss alternative trial plans versus whether or not there appears to be sufficient information for submission of a license application.

Now the end of Phase 3 trial may or may not incorporate the aspects of a pre-IND, a new drug application or pre-BLA, biologics license application meeting. The focus of that type of meeting would actually be to determine the required contents and format of a license application to insure that the application will be complete and fileable upon receipt. The other aspect would be to insure that the organization will allow an efficient review process.

If indeed a license application is filed, another aspect of meetings with the Agency would be at the time following an advisory committee meeting to discuss the recommendations of the advisory committee and to discuss whether or not there are needs for additional trials or data either prior to licensure or following the licensure as post-marketing commitments.

With regards to a license application or drug application review process, there are specific time lines which have now been codified. At the time of receipt of the application the Agency has 45

to 60 days after receipt depending upon the status assigned to it, to determine whether or not the application is indeed materially complete, well-organized and reviewable.

During this period, the assignment of the review designation is made. And there are two reviewed designations which may occur. The first is a priority review which would be a 180-day period in which the Agency would review the entire contents of the application and develop an questions, comments or requests for additional information. This priority review assignment would be designated if the underlying disease being treated was serious and life threatening and that the drug itself was treating a serious aspect of that disease and represented a significant advance in the treatment of that disease.

Other applications would receive a standard designation which would mean that the review process of that application would be completed within a 10 to 12 month cycle. For standard applications, we are, as an Agency moving towards a 10-month cycle over a period of several years.

There should be on-going communication between the review team and the sponsors for easily addressable questions during the review process. While major requests for information may not always occur during that period of time, definitely any areas which could be clearly easily addressed in communication would be handled and there is for most new applications new chemical entities, new biological entities an advisory committee presentation at the time of the initial approval and depending upon the therapy, the aspect and any other particular areas where the Agency feels they need guidance of the scientific expert committee.

Again, we reviewed that there are several times when the Agency would ordinarily meet with the sponsor. There are several ways in which the Agency would communicate including specific written requests and also telephone and video conferences are options for communication during the process.

David? Next slide. And again, the basis for approval is clearly that there be replicable demonstration of efficacy with acceptable safety and adequate and well-controlled clinical trials. And the entire license application should

allow one the ability to write a product label that defines an appropriate patient population for treatment and provides adequate information to enable safe and effective use of the drug.

Now there are some specific provisions for expediting drug development or facilitating drug development which the Agency has codified again in the regulations. The special provisions for serious and life threatening diseases in which cancer would clearly fall are the expedited review or Subpart E process accelerated review and both of these have been subsumed into the new legislation of the FDA Modernization Act which also incorporates fast track as a review process.

Expedited review is actually a process which occurs under the investigational new drug development phase, so prior to licensure and submission of a license application, expedited review is a little bit something of a misnomer. It is more like an expedited or facilitated drug development process where there is clear evidence of activity in the early phases of disease. It is intended for serious and life threatening diseases and by this we mean serious and immediately life threatening diseases often. The procedures are in

place to expedite the development process, to take something which has already shown evidence of activity even in the earliest trials and to rapidly bring it into the definitive trials which will determine, which will confirm that evidence of effectiveness as well as provide additional safety data.

The regulations encourage early and repeated contacts with FDA staff. It provides for the potential for marketing approval based upon an adequate efficacy demonstrated in an adequate and well-controlled clinical trial. So one thing this does not do is lower the standards for efficacy. It basically shortens the drug development process. And it says this could be done in Phase 2 trials were appropriate in the setting where a reference group or natural history of the diseases well are known, otherwise it would imply that this would be done in a controlled trial.

And further evaluation would typically be conducted under Phase 4 studies as a rapid development process might leave many holes in our understanding of the entire safety profile or in less common aspects of adverse events.

The accelerated approval process is present both in the biologics regulations and in the drugs regulations for the marketing. It is again intended for serious and life threatening diseases in areas where therapy appears to provide a meaningful therapeutic benefit over therapies which are in existence for treatment of that aspect of disease.

The approval would be based upon a surrogate end point or an end point other than survival or irreversible morbidity. The approval is conditional which means that if subsequent studies show that the evidence of efficacy has not been well established, the approval itself may be withdrawn. The approval may carry additional restrictions to insure safe use which would be determined based upon the safety profile available at the time of approval and whether or not there are needs for additional limits on use of the drug until further data are available to suggest that it could be expanded or lifted.

The FDA Modernization Act of 1987 incorporates many aspects of these in one location in the regulations. It is intended for this aspect of facilitating drug development, again, is intended

for serious and life threatening diseases which demonstrate the potential for unmet medical need. In such diseases one may -- a drug developer may request fast track designation for their product which incorporates again many aspects of considerations of expedited development and facilitated development as would be appropriate given the data available.

There should be -- there is under this designation a condition for a rolling application which is to file completed sections of the application as they become available, rather than at a single point in time which may facilitate the application submission to the Agency, may allow the Agency, if time permits, to review the application more rapidly and however, the fast track designation does not clearly imply that rolling BLA submissions or rolling NDA submissions will, in fact, occur. That is made as a separate decision at the time when sufficient data are available. And applicants who were interested in finding out more about fast track for tumor vaccine drugs may contact Bette Goldman at the Center for Biologics at this number.

Now in terms of tumor vaccines, clearly this product class is different from traditional

cytotoxic agents. And as such, one might need to consider what aspects of the development process really need to be handled a little bit differently and these are some thoughts as to where one might consider some flexibility or some different approaches in the area of drug development.

The first would be in the objectives of the Phase 1-2 study. As you've already heard from Dr. Liu and will likely hear from others, the identification of a Maximum Tolerated Dose which is the goal of most cytotoxic agents is very unlikely to be the goal for tumor vaccines and clearly, the identification of a pharmacologically effective dose or an optimal biologic dose would be a much more reasonable goal in this field. The rationale for this would really be two-fold, the first being that biologically active doses may well occur below the maximum tolerated dose and also in terms of feasibility it's technically infeasible often to even determine what that maximum tolerated dose is, so that even if one were interested in determining what could happen in a worse case scenario it's just not possible to do it.

Next slide. In terms of design issues, the dose selections which are going to be evaluated

in the initial studies should really cover a very broad range to characterize the relationship between dose and immunologic activity. Rather than in the traditional dose escalation paradigms, what we would like to see is a proposal which incorporates both the potential for toxicity based upon prior knowledge of the drug class in preclinical studies for toxicity and also for the potential to observe the actual differences between dose cohorts so that what one is really trying to do is not so much evaluate the differences in the toxicity level, but the differences in the immunologic activity level between dose cohorts. So we may consider in drugs which appear to have a very safe profile for toxicity, if that's not an oxymoron, that a wider dose escalation than one would consider for traditional cytotoxics would be much more reasonable to employ.

But critical to this would be the fact that we need to have assays which are able to discriminate the immunologic responses between different dose levels so we have to have assays which are not just an on-off, but might actually be able to distinguish between different levels of an immunologic response.

Next slide. Again, as you've already heard, the --I'm sorry, go back one. There. The patient populations for these studies likely will be somewhat different. Immuno competent patients are necessary in order to be able to observe the immune response. Unfortunately, we don't really have a good handle on how one defines an immuno competent patient and I fully concur with Dr. Liu that it would be well to try and elucidate what factors are important for identifying patients who have a like level of immunocompetents, what level, what factors might identify patients might be used to identify such patients. At this point in time, it's generally looked on as patients who have not been heavily treated or have other very gross parameters of immune competence and the more precisely we can elucidate that, characterize it and characterize patient populations more likely we will be able to compare cross studies.

The underlying disease should not be a rapidly progressive one so unlike the traditional cytotoxic studies which are done often in end stage patients or refractory patients, we need a population that's going to be able to remain on study long enough to receive sufficient exposure to

develop an immune response. And it may be reasonable to conduct this either in very early stage of disease or even in the adjuvant settings often from almost the initial studies.

Measurable or evaluable tumor has generally not been considered necessary because one frequently with this class of products has not expected to see evidence of anti-tumor responses. The Agency is not meaning to discourage those people who wish to look in that area, but if that's clearly not the goal of the study, it's not necessarily a useful restriction to place on eligibility.

The other issue is that one might rather than do the traditional three to six patients per dose level cohort, consider again to justify the size of the dose level cohorts based upon the number of patients and the amount of information necessary to be able to distinguish between groups of patients and cohorts. If three patients isn't enough, if six patients isn't enough, do 10, do 12, whatever is justified based on the assay system that will be able to detect differences between the dose level cohorts and these sorts of things should drive the design of the studies.

Next, please. In terms of the analytic methodology, again traditional cytotoxic drugs that look at an NDT prediction generally have very limited and descriptive analytic methods in their early trials. We would expect for this product class that there actually be much more description, both of the analytic methodology and the basis for many of the aspects of the trial design to determine whether or not it's really a reasonable approach.

We would like to see detailed description of the immunologic assays to be used in the study as the basis for determining the initial surrogate of activity, including the controls and performance characteristics of those assays. We would like to see a basis for the number of patients per dose level and the overall sample size and we would like and this seems like it should be an understood thought, but we really need to know what it is that people think is the measure of success of biologically active or optimal biologic doses based upon the immune response.

In the Phase 2 studies, again, continued exploration of dose schedule, route of administration, a variety of factors. And to highlight what Dr. Liu also said, the exploration of

some of these aspects might best be done in a randomized Phase 2 study that compares the multiple strategies contemporaneously. This eliminates the confounding variables that we see in a series of Phase 2 studies would each study one small aspect. It allows us to make comparisons across groups when they're randomized and entered. It removes one additional level of complexity and confounding nature between the groups.

Among those multiple strategies which we would encourage to be explored would be approaches with various immuno adjuvants, cytokines, multiple antigens, but we think that it's profitable to actually explore those contemporaneously and across groups.

Next. Finally, for the Phase 3 studies, these products have generally been conducted in -- been evaluated in the adjuvant setting or in minimal residual disease states and because of this, the studies have generally looked at survival, progression- free survival and these types of end points need to be evaluated in internally controlled and randomized trials. Again, because of the confounding factors of comparing a cross study to reference group, historical controls, we have, we

would say as a general statement that for studies look at survival and progression-free survival that these need to be randomized internally controlled studies.

The efficacy standards again, I can't say this enough, adequate and well-controlled trials. For the initial approval, if the setting is one of an adjuvant disease one of a minimal residual disease state, and if the body of evidence from the prior trials really doesn't show any clear evidence of clinical benefit as one might see with tumor responses in traditional cytotoxic chemotherapeutic drug development, then it's likely that more than one Phase 3 or controlled randomized trial, whether one calls that Phase 3 or not, will probably be necessary. However, for supplemental approvals, one could use the activity in the initial disease setting and the initial approval setting to be supportive of activity in a second setting and we are open to creative suggestions as to how to consider multiple trials which replicate these effects.

An initial about surrogate end points and I think needs to be addressed. The Agency has recognized that durable complete and partial tumor

responses are acceptable as surrogate end points in most, not all, but most malignancies, advanced malignancies.

Time to progression has been accepted in a surrogate in some areas including adjuvant settings and the hormonal therapy of prostate cancer. On occasion, this has also been considered an outright measure of efficacy, but it depends upon the setting.

Next slide. But one thing that needs to be understood is that effects on serologic tumor associated antigens, that is, effects on CA-1 25 level of PSA, for instance, have not been considered to be surrogate end points which would be sufficient to demonstrate efficacy. Effects on reduction in cells containing a gene marker for disease to some area below the limit of detection has not been considered to be an end point which is reasonably likely to predict clinical benefit. Clearly, immunological responses against tumor antigens has not been accepted as a measurement, as a surrogate which is reasonably likely to predict clinical benefit. That is not to say that such end points may not ultimately be validated, but at this point in time they are not considered to be areas which we

would accept as an Agency as an end point which could support efficacy as the primary evidence of efficacy for clinical trial.

I think that -- no, skip that, Dave. I think I'm going to skip some of these in the interest of time. Okay. Yes, that's it. And now I think I will introduce the next talk which will be Dr. David Essayan from the Division of Clinical Trials, Design and Analysis who will be speaking on the preclinical safety and efficacy studies.

(Applause.)

DR. ESSAYAN: I'm just loosening up the mike. Can everybody hear me with this? Yes. Okay. I'm sort of allergic to podiums. Let me just give it a shot from here.

Well anyway, this is the preclinical development part of vaccine programs. I'd like to introduce you to the Pharmacology Toxicology Branch in the Office of Therapeutics at CBER. This branch does all the clinical pharmacology, as well as all the toxicology for the Office of Therapeutics. We also function in a consultative function to the Office of Vaccines, as well as the Office of Blood. In addition, a number of the medical officers here do clinical review in their fields of expertise.

But for Pharm Tox issues, these are the folks that you're going to wind up speaking with.

We see a number of different strategies in the Office of Therapeutics in the context of tumor vaccines. I'd like to go over a couple of them, briefly. Obviously, modified and unmodified autologous or allogeneic tumor cell products. There are also soluble tumors, associated antigens in the absence or presence of a wide variety of adjuvants. The tumor-associated antigens themselves can be of a variety of different sources. They can be recombinant, they can be purified. There are a number of different types of tumor-associated antigens that we have seen.

Additionally, we see tumor-associated antigens of all these varieties and combinations loaded into a variety of antigen-presenting cells including the dendritic cells that have been discussed and will be discussed further during this conference. There are gene modified products such as tumor cells and then what I group here as "other", oncolytic viruses, viral vectors for gene therapies, etcetera. Despite this wide variety of different product, our general goals in the preclinical safety program remain remarkably the

same. The first goal is to recommend an initial safe starting dose and dose regimen in human subjects.

Now here it's important to note that the safety is a function of each component of the vaccine as well as the interaction of the component such as with a tumor-associated antigen and the adjuvant selected. The preclinical study should help to define not just the dose activity relationship, but also the dose toxicity relationship, the differences between these two both in terms of dose and in terms of organ and tissue specificity as well as the effects of route and schedule of administration on the activity and toxicity. An example of this would be an intradermal or IV versus sub-cut administration where different antigen presenting cells in different mechanisms may actually be invoked.

The second goal of the program is to identify potential target organs for toxicity related to the product. In vitro tissue binding and/or target antigen distribution studies, whichever is appropriate for the individual product, are a critical first step. These studies may guide gross and histopathologic studies which may, where

appropriate, guide subsequent safety pharmacology studies which will look at specific organ related toxicities. These studies should help to define the dose dependence of the toxicity, the relationship to exposure and importantly, the potential for reversibility of these toxicities.

The third goal is to identify appropriate serologic and immunologic parameters for monitoring safety and efficacy of the product in human subjects. Now this will be discussed quite widely during the two days of this conference. I would just like to say that the quality, quantity and relative contributions of the cellular arm, the humoral arm as well as the potential role of complement may be delineated in the toxicology program and may be correlated to outcomes in the preclinical models which may provide helpful insights for development of the product.

The fourth goal is to identify potential at-risk populations for administration of the product. Such identification may be guided by both target organ toxicity data and the outcome of product administration in the context of animal models of disease. Now there are a number of pros and cons with animal models of disease. The cons,

obviously, there's often a limited historical data base for the background on the animals. Oftentimes, these animals are quite ill and interpretation of toxicologic data in that context may be challenging. However, in animal models of disease, good, strong animal models of disease, these data may provide relevance for specific disease states that are difficult to come by other mechanisms and so I would encourage you to consider this.

The next goal is to help determine an acceptable risk benefit ratio for human subjects. Now risk benefit may vary according to the indication as well as the intended target population and in fact involving pre-clinical and clinical experience with the product may over time shift the risk benefit ratio during product development.

And the last is to help elucidate the mechanisms of action of the product. An optimal dose regimen needs to consider both the immunogenicity of the vaccine, the specific immune response desire related, in part, to which arm of the immune system is felt to be most important for the biologic activity of the product and importantly, as Dr. Keegan and as Dr. Liu have

alluded to, the immune status of the subjects to be studied.

What types of preclinical studies are available to us? Preclinical studies that should be considered for all vaccine products include local tolerance studies, pharmacodynamics which in this particular context may be translated into immunogenicity studies, safety pharmacology where organ specific toxicities have been identified in your single and repeat dose toxicology studies and can be pursued. If there is suspicion for a particular organ related toxicity for that individual product, in fact, the safety pharmacology studies may be incorporated into other single or repeat dose toxicology studies.

Other studies, other preclinical studies that should be considered where appropriate for specific vaccine programs include ADME studies, particularly related to some of the viral products that we see; pharmacokinetics, carcinogenicity, genotoxicity and reproduction and developmental toxicities where applicable to the individual product.

There are two relevant ICH documents that may help guide our view of the preclinical

safety program, the M3 document and the S6 document. I should note that the S6 document does not specifically cover cellular and gene therapies.

The M3 document states that toxicology should be performed in two relevant mammalian species, one nonrodent with a dose intensity that is greater than or equal to that anticipated in the clinical trials. It further states that where appropriate ADME genotoxicity, local tolerance and certain carcinogenicity studies should be performed prior to the initiation of Phase 1. In the context of tumor vaccine, I would particularly focus on local tolerance studies.

Reproduction and developmental toxicology should be conducted as appropriate for the population that is to be exposed and for the product. Special consideration for pediatric administration including the availability of reproduction and developmental toxicology, genotoxicity, carcinogenicity and potentially studies in juvenile animals in order to target the developmental stage in your preclinical program.

Having said those things a step-wise development program is acceptable, so called rolling toxicology and importantly the safety evaluation may

be considered on a product specific basis if existing paradigms are either inappropriate or irrelevant in the context of the particular product.

The S6 document reiterates a number of these major issues. Preclinical safety testing should consider selection of relevant animal species, the age of the animals, the physiologic stage of the animals, normal versus disease models, the delivery as well as the stability of the test material under the conditions used.

The routed frequency of administration should parallel as closely as possible that proposed in the clinical trial. Optimally, exposure to the product should define a no observable effect level, a no observable adverse effect level, the pharmacologic effect and as Dr. Keegan has mentioned, the MDT is often less critical in the context of immune response as compared to the optimal biologic dose. In the preclinical models, however, MDT determination, if possible, may give you an idea of the window that you have above optimal biologic dose. Where appropriate, safety pharmacology can be incorporated into the design of toxicology studies.

Studies should try to include a recovery period for assessment of late toxicities and potential reversibility. I'll get back to this issue on a subsequent slide.

And again, a flexible science based approach designed to address issues specific or unique to each product should be utilized for the pre-clinical safety evaluation.

What of our major concerns looking at any new application in the Office of Therapeutics? Injection site reactions are very commonly seen while most of these are minor, some of them can be quite major. We have actually seen grade 4 local toxicity related to certain vaccine related products and so this should be one of the major focuses of the toxicology program.

Induction of autoimmunity is often discussed and here we need to think in terms of the antigen specificity for the individual vaccine product, its distribution and the concept that the majority of these vaccine protocols in one way or another are seeking to overcome self-tolerance for that individual antigen or group of antigens. At the point when efficacy, when biologic activity is established, one has presumably overcome in some

measure tolerance to self. At that point, the threshold for generation of an autoimmune response based on the natural distribution of that antigen is theoretically possible and should be a focus of the toxicology program.

Hypersensitivity to vaccine components, we have seen this rarely, but it is of some concern to us when we do see it. Systemic toxicity and pyrogenicity are seen with a variety of these products, as is regional lymphadenopathy following administration of the product.

Cytokine release syndrome has been seen with a number of these products. This can be a severe and in fact life-threatening complication and should be monitored preclinically and also if seen then specific considerations in any clinical trials be undertaken. The last consideration here is the potential for induction of disease which will be discussed in some detail later on during this workshop.

Major limitations to the preclinical studies include species specificity and by this I mean to denote both variations in the immunophysiology between the preclinical species

chosen and humans as well as species differences between the tumor tissues being studied.

Two other aspects are a direct outgrowth of the direction of most toxicology studies, that being difficulty in modeling long-term toxicities. This can be, in part, related to immunogenicity of the product that is counter regulatory, so to speak to the biologic activity of the product in your model, in your animal model, as well as difficulty in adequately assessing the potential for reversibility of the toxicity.

So having made these points my conclusions would be that the preclinical program needs to address the safety and biologic activity of the product as well as the mechanism of action of the product and that unique properties of individual products must be considered on a product-specific basis for the preclinical program.

Thank you very much for your attention.

(Applause.)

DR. KEEGAN: Okay, our next speaker will be Dr. Raj Puri who will be speaking about product development issues.

DR. PURI: If I may have the first slide, please? First slide, please? Can somebody turn the slide on, please?

Well, while the slides are being turned on I will continue, I guess.

After you have heard general and clinical issues that should be considered for tumor vaccines, clinical trial development from Dr. Keegan and types of preclinical studies that should be considered from Dr. Essayan, my task is to summarize what should be done or considered for product development for tumor vaccines.

Before I do that I would like to list some of the types of tumor vaccines as soon as the slides turn on, but if they don't, I will continue. One of the types of tumor vaccines are cellular tumor vaccines and the second class is multi-antigen preparations. The third type are purified proteins, synthetic peptides and other gangliosides. The fourth type could be a type as vital and plasmid vectors which could be injected into a patient within or outside the liposomes.

Cellular tumor vaccines which is my first slide is characterized --

(Laughter)

Cellular tumor vaccines are comprised of autologous tumor cells or allogenic tumor cells and these tumor cells could be unmodified or maybe modified by chemical agents such as dinitrophenol or they could be irradiated before they're injected into the patient to boost the immune response.

These tumor cells may be also -- there it is. That was my first slide.

(Laughter.)

May I move to the second slide? These are some of the types of tumor vaccines that are indicated, cellular vaccines, multi antigen preparation, purified proteins, synthetic peptides. Others such as gangliosides and vital and plasmid vectors, and the liposomes.

Cellular tumor vaccines as I already said could be autologous allogeneic tumor cell, they could modified with a DNP and combined with an adjuvant or growth factors before they're injected into patient to boost immune response. These tumor cells could also be genetically modified to secrete factors, cytokines, chemokines and surface expression of MHC antigen and other co-stimulating molecules before they're injected to the patient to boost immune response.

Other cellular tumor vaccines include lymphocytes that could be derived from peripheral blood or from the lymph nodes and other cells are antigen presenting cells or antigen pulse dendritic cells, fibroblasts or other cells and these cells could also be derived from peripheral blood or from the bone marrow.

These cells could be pulsed by a variety of different agents such as RNA, tumor cell lysates, synthetic peptides, multi-antigen preparations and so on. And in some situations these cells could be co-cultivated by another cell such as Orosophila cells which are designed to express an immune stimulatory molecule such as IKN-1 and other immune obligatory molecules.

Multi-antigen preparations include tumor cell lysate, cell tumor antigens or secreted tumor antigens, tumor antigens which are either conjugated with KLH or as such.

These preparations could be injected to the patient as such. They could be mixed with the adjuvants or in some situations they could be used to pulse antigen presenting cell such as a popular dendritic cell.

Purified proteins and peptides and others such as ganglioside, GD2, GD1, GM1, GM2, these antigens either could be purified from a cell source or they could be produced by recombinant DNA technology. These antigens, heat shock proteins, idiotypic, antiotypic antibodies and fusion proteins such as heat shock protein and peptide complex that could be used as such are mixed with the adjuvants before they're injected into the patients. In some situations, these products would also be used to pulse antigen presenting cells such as dendritic cells.

Synthetic peptides and gangliosides, they could also be mixed up with the aduvants or KLH and in this case where KLH ganglioside conjuvants could be mixed with the adjuvants such as suponin or with cytokine and injected into patients before -- to boost their immune response and synthetic peptides could also be used to pulse different types of antigens presenting cells.

Vital and plasmid vectors also qualify as tumor vaccines. Vital vectors may include vaccinia virus, canary pox, fowl pox, adenovirus, adeno associated virus, herpes simplex virus or what have you.

These vectors or plasmid vectors could be designed to express many different factors, cytokines, growth factors, tumor antigen, viral antigens or some are tumor associated antigens.

The issues associated with this kind of tumor vaccine will not be discussed in the current workshop. Issues related to this kind of tumor vaccine have been addressed in the prior FDA and NIH workshop and they are addressed in the available FDA guidance document termed Cell and Gene Therapy.

Many of these products and peptides are encapsulated in liposomes or they're mixed with the lipids and thus they form another type of tumor vaccines and they're injected into patients to boost the immune response.

For all types of tumor vaccines or any other biological drug, general regulatory principles apply as was emphasized by Dr. Zoon. Cell substrate and cell bank characterization of this product should be thoroughly characterized. Their regulatory guidance document available from the Agency that should be consulted for this particular purpose. This particular issue will also be discussed later on in this session by Dr. Allen

Albright of Center for Biologics Evaluation and Research.

For a typical biological drug, identity, purity, and potency and safety should be established at the very stages of the product development. And this would continue into Phase 3 studies as I will discuss later on.

The potency is defined as measurable, consistent biological response to vaccine in vitro or in vivo in the animal model.

For reproducible and consistent product and to maintain lot to lot consistency, the manufacturing process would be controlled and all the glitches should be hammered out before undertaking higher phase of the clinical trial program.

In the next two slides, including this one I will summarize the types of studies that should be performed in early and late stages of tumor vaccines product development. At early stage of the product development such as Phase 1 or Phase 2, the major issue here is safety. To address this, the products should be characterized thoroughly for the freedom from the adventitious agents that include viruses, sterility includes bacteria and

fungi, mycoplasma and the endotoxin content must be within the allowable range. The allowable ranges less than or equal to five endotoxin units per kilogram per dose.

Lot release specification should be set at this particular point. The source of raw material, the components used in the manufacturing process and the process itself should be very well characterized. Although complete identity and potency tests are not required at the Phase 1 stage of the tumor vaccine development, but they should be -- the development should begin after, if needed, after consultation with the Agency.

The stability program should typically include integrity, quantitative identity tests for products such as mixture of cells and functional activity which is potency. The integrity of the product could include measurement of viability of cellular products.

At later stage and before embarking at the pivotal of Phase 3 studies all assays for the determination of identity and purity and potency should be ascertained and they should be validated. The lot release specification should be tightened

and this would continue to be developed during the entire product development.

For validation studies, removal of all in-process reagents, examples, cytokines, growth factors, antibodies or enzymes should be completed or on specific occasions should be set if these cannot be removed completely.

The stability program should be completed to support the proposed dating period for Phase 3 clinical studies. We also recommend at this stage that you set up a pre-pivotal meeting with the Agency to discuss the product and manufacture and the clinical issues.

Next I will discuss about some of the important issues related to various classes of tumor vaccine. For autologous tumor vaccines process of generation of single cell suspension from solid to tumor chunks may define the product and thus, it is very critical. Since tumor cells must be digested with different enzymes for various concentrations of enzymes at different temperatures and different times, thus it is very critical to identify and look at the viability of these cells and set up some sort of specifications.

Since tumor nodules may have some infiltrating components it is therefore very important to characterize the cell types in the cell suspension. The sterility of these products is very, very critical for the safety of the patients. No product should be injected if it is contaminated with any of the advantageous agents that include bacteria, fungi, and mycoplasma if these cells are cultured.

If these cells are cultured, the mycoplasma contamination should also be tested as fetal calf serum and other incoming reagents and processes in the cells may introduce mycoplasma contamination.

If there is not enough time to determine the mycoplasma contaminator, contaminants that you may explore alternate faster technique such as polyvalent chain reaction or PCR. In conjunction with the standard tests during early phases of the development, in order to collect information on the usefulness of the PCR test, potentially these assays can be validated and if found compatible they can replace conventional tests.

The purity of the final cell preparation should be defined. Freedom from endotoxin agents and in-process agents should be ascertained.

One of the very important tests for this class of product is potency. It is very difficult to assign potency in this situation because all patients' product is a unique product and thus it is a difficult issue. Should presence of certain phenotype of cells in vaccine preparation and generation of in vitro immune response to vaccine must be performed before tumor cells are injected into the patient is a question and that should be addressed.

These issues will be discussed in tomorrow's session No. 3 and I hope they will be able to reach some sort of consensus to the questions that we have raised and we have provided to you in your program book.

If the cells are shipped, the shipping condition must be validated and confirmed to determine that the shipping conditions have not changed the cellular phenotype of your product.

Similar to all biological products, the stability program for autologous tumor vaccine must be established particularly if repeat administration

of the cells at plan. It is important to know whether you are going to inject same vaccine on second cycle, third cycle, or so on as you inject it.

There are some important issues associated with allogenic tumor cells. Unlike autologous tumor cells in this case one has enough time to characterize the product completely. The most important issue with allogenic tumor cell vaccine is the donor screening and saline characterization. These salines may be obtained from ATCC or they may be derived in your facility. They must be fully characterized for freedom from advantageous agents such as viruses, bacteria, fungi and mycoplasma.

Quantitative assays should be established to determine product identity, more importantly in situations where more than one saline is mixed and used as tumor vaccines. The potency should be assigned to the product and must be established before embarking on Phase 3 studies. Again, if these vaccines are shipped and handled at different clinical sites, their shipping and handling conditions should be validated to maintain product integrity. These issues will also be

discussed in tomorrow's session No. 3 and I hope that you will have various input so that you can help the Agency in deciding some of those tests.

Generally, dendritic cells, antigen-presenting cells and dendritic tumor fusion cell vaccines are derived from autologous source and their phenotype may vary depending on the cell source. Furthermore, in vivo mobilization by flit three ligand may enrich a different population of cells. Therefore, phenotypic characterization of these cells and determination of antigen load form an important issue when characterizing identity of this class of tumor vaccines.

These issues will be discussed in today's afternoon session and I hope there will be some sort of a consensus on agreeing to the prominent phenotype that should define dendritic cell.

The other important issue associated with this class of tumor vaccine is potency assay and that is determined by antigen presentation and biological response which is ability to induce immune response, for response, proliferation of responder cells, generation of CTL response and production of cytokines.

Before embarking on Phase 3 program studies should be finalized to decide whether they are actually activated and are actually presenting antigen. These issues will also be discussed in today's afternoon session and I hope there will be some sort of agreement to agree how to define the potency of activated antigen presenting cells, dendritic cells or like cells.

For tumor cell lysates and polyvalent vaccines characterization of cell source is very critical. If allogeneic tumor cells are used, donor screening of the saline and for the safety should be emphasized as it was done for the allogenic cells when they're used as tumor vaccines. Like autologous tumor cells the manufacturing process for the generation of cell lysates and shed soluble tumor antigen is very, very critical.

The identity test for this class of product is critical as identity tests for difference cell mixers used at tumor vaccine. It is important to define the quantitative presence of certain known tumor antigens in the lysates or tumor cell mixture or antigen mixture.

The characterization of this class of product, tumor cell lysate and polyvalence tumor

vaccine should be performed by a number of available techniques. For example, cell number determination, viability from where the cells -- where the product is derived, total protein concentration, SDS-PAGE including Western blot analysis for known protein or peptide, 2D-electrophoretic patterns so there are not too many proteins secreted by the tumor cells and gel filtration patterns.

These are only just an example and the test will vary depending on the product. You could apply any of the techniques which you might have to characterize these kind of products. Potency, like with any other product this issue is critical here as well. An assay that can determine consistent biological response to these products in vitro or in vivo in animal model should be desirable.

These issues will also be discussed in tomorrow's session and also in a poster session that will be held after the Session 2 today from 5:30 to 8 o'clock and will continue until tomorrow. Your participation in this session, your considerations and your thoughts on this issue will be of great value to the Agency.

As with recombinant of purified protein some of the required characterizations of this

class, purified protein antigens of tumor vaccine include purity, potency, identity and safety by available analytical tools. And some of the available analytical tools are for example identity could be defined by end terminal sequence.

The purity could be defined by SDS-PAGE, tryptic digest or HPLC patterns. The potency of this class of products could be defined by generation of CTL response, cytokine secretion of proliferation assays of the pulse cells. This kind of tumor vaccine, the safety by the freedom from infectious agent that includes bacteria and fungi and endotoxin and other process, end process regions should be assigned.

Unlike tumor cell lysates synthetic peptides could be easily characterized by available techniques. Some of the most common techniques that are used are spectrophotometric analysis, that includes infrared, MNR and mass spectroscopy. Amino acid analysis and complete sequence analysis can define the identity of this class of product. Purity could be defined by HPLC or high performance capillary electrophoresis. In some situations the complete sequence analysis can determine the purities. Organic solvents such as chloroform and

acetonitrile are commonly used to synthesize and purify these peptides. The validation of removal of organic solvents for safety is critical for this class of compounds even before embarking on Phase 1 studies.

If peptides are used as such and when they are mixed with adjuvants the potency of this class of tumor vaccine should be defined on peptides themselves or the peptides that are used to pulse the dendritic cells, the potency should be determined on the pulsed cells.

For peptides or plasmids or vital vectors that are encapsulated in liposomes there are important issues associated with them. For example, the composition and the source of the lipids and the pH of the liposome mixture can determine the optimal encapsulation of your product that could be an antigen, that could be DNA or it could be a peptide.

The particle site and the viscosity and the residual solvents should be determined for the safety. Like all products, the stability of liposomes at storage temperature should be determined for later phases of the product development.

I have listed here some of the telephone numbers, fax numbers, e-mail addresses and the internet site from where you can obtain various regulatory guidance documents that are available to you for product development. These addresses and the list of relevant documents are provided to you in your program book. Of course, you are invited to call us any time you have any questions regarding tumor vaccine product development. We'll be happy to attempt to address your questions that you might have.

I would like to acknowledge some of my colleagues from NCI and the FDA who have helped me, particularly Dr. Jay Greenblatt. We have a wonderful collaboration in setting up this workshop. Dr. Earl Dye and Dr. Joyce Frey-Vasconcells from Center for Biologic Evaluation and Research who have given me a lot of input in preparation of this talk and of course, members of Tumor Vaccine Workshop Organizing Committee for their valuable contribution in organizing this workshop.

Finally, I'd like to thank you for your participation and your kind attention. Thank you very much.

(Applause.)

DR. KEEGAN: Okay, in organizing this meeting one of the things that we had discussed is the Office of Vaccines has many, many years of experience with vaccines in relation to infectious disease. So the next two presenters are going to provide us lessons that they've learned with respect to infectious disease and hopefully this will provide you some insight and some guidance into issues that you can think about in relation to tumor vaccines.

So the first presenter is Dr. Donna Chandler and she's going to talk about lessons from preventive infectious disease vaccines in relation to bacterial vaccines and adjuvants.

DR. CHANDLER: Can you hear me? Is this going to be okay? There we go. Thank you, David, for your help.

I'm Donna Chandler. I'm in the Division of Vaccines and Related Products Applications in the Office of Vaccines Research and Review and I did include a phone number and you're welcome to call if you have specific questions about bacterial and viral vaccines.

This is an outline of what I'd like to try to go over with you this morning. I'd like to

give some examples of preventive bacterial vaccines, talk a little bit about preclinical studies and vaccine adjuvant issue, go over some of the clinical data that we expect to see for vaccines and then finally help with -- give a list of some of the common pitfalls that we've seen. I'm talking from the experience with bacterial vaccines and most of what I'm going to say, in general, applies to viral vaccines as well and then Dr. Albright will focus on viral vaccines and cell substrate issues.

This is a list of the preventive vaccine examples. We have things such as toxoids, such as diphtheria, tetanus and pertussis toxoids, inactivated bacterial vaccines such as whole cell pertussis, purified antigens such as pertussis fimbria hemagglutinin, the 69K protein or protastin, typhoid polysaccharide the VI polysaccharide is a purified antigen and then the pneumococcal polysaccharide antigens in the 23 valent pneumococcal vaccines.

We also have examples of live attenuated bacteria such as the salmonella typhi Ty21a. This is actually the only oral bacterial vaccine that we have currently approved.

There are recombinant proteins such as the lyme recombinant outer surface protein A. We also have conjugate vaccines. A success story with the hemophilus type b PRP-CPM, polysaccharide conjugated to protein. We have a number of these types of conjugate vaccines approved and then I would also like to mention combinations that it is feasible to combine a number of vaccines such as the DTP whole cell pertussis, hemophilus conjugate which is tetramune and hepatitis B hemophilus convax.

Now let's see, okay, in terms of therapeutic vaccines currently we don't have any therapeutic vaccines approved for an infectious disease indication. BCG Live is approved for bladder cancer but that probably works by a nonspecific stimulation of immune mechanisms.

This is kind of an overview slide of what we would expect to see in terms of a preventive vaccine development. We would like to see, of course, clinical data on safety as well as efficacy data. We would expect to see information on manufacturing consistency. There are specific Code of Federal Regulations requirements such as potency, sterility, purity, identity and we're still mandated

to use the General Safety Test for vaccines unless a specific exemption is requested.

Stability is important to establish an expiration date. The package insert or labeling has to be developed and the safety and efficacy data would be presented at the Biological Products -- Vaccines and Related Biological Products Advisory Committee and of course, a pre-licensing inspection of the facility would be required.

I'd like to move a little bit now to -- whoops, I think we skipped one. Here we go. Okay, fine, thanks.

I'd like to talk about the vaccine preclinical studies. I don't want to go over the same sorts of things that Dr. Essayan presented, but they differ a bit from classical drugs or even therapeutics in the Office of Vaccine. For one thing, most vaccines have limited toxicity and they're given in limited doses, generally, maybe one to five doses over months or years. But in terms of preclinical data that we would expect to see, we would like to have some information on potency and immunogenicity. Immunogenicity is a very important aspect of vaccines and we rely on it a great deal. Pyrogenicity, the rabbit pyrogen test or LAL test

for endotoxin, in some cases the CRF actually exempts bacterial vaccines from pyrogenicity, but we usually see that as a test.

Challenge and protection studies in an animal model. If an appropriate animal model exists is important and useful data for us. For live organisms which have been attenuated, the documentation of the level of attenuation is important. For bacterial toxins which have been inactivated, we would want to have information, expect to see information to show that that inactivation is complete or to document the extent of inactivation and also to show that reversion does not occur on storage.

Private characterization issues would include the necessary aspects such as, for example, a polysaccharide conjugate vaccine. We would want to see information on the ratio of the polysaccharide to the protein carrier and perhaps any information on the percent free polysaccharide.

It's becoming more relevant that we would expect to see good laboratory practices safety study to support a Phase 1 clinical trial. And while this is still developing or this policy is still evolving, I think you would expect to see more

requests for ReproTox studies for vaccines that were intended to be used in adults.

I'd like to go over quickly some of the adjuvant issues and in this case I'm using adjuvant as defined as an agent that augments specific immune responses to antigens. Currently only aluminum compounds, alum, aluminum phosphate and aluminum hydroxide are the only compounds, the only adjuvants approved in currently licensed vaccines. These specific antigen adjuvant formulation is licensed and adjuvants alone have not been approved for generic use with vaccines.

There are a couple of references I'd like to refer you to for preclinical studies for vaccines with adjuvants in AIDS Research in Human Retroviruses and there's also a list of about 80 products in a compendium of vaccine adjuvants in exipients in the book Vaccine Designs, Subunit and Adjuvant Approach. And this was compiled by Fred Vogel and Mike Powell.

This contains a list of structures, uses, chemical and physical properties and safety and toxicity in the adjuvants.

Okay, I'd like to kind of go through quickly some of the principles for toxicologic

studies of adjuvant and vaccines. Basically, our experience was aluminum compound supports the safety. In other words, we wouldn't require additional studies on the aluminum adjuvant alone. However, if an adjuvant, a novel adjuvant is being used, we would expect a single repeat dose toxicity study and as we get, see more adjuvants and we are moving toward the realms of studies that need to be, that have been done and are required for the more classic drugs.

The principle again, as you've heard and will probably hear again, the preclinical study that you choose, the preclinical information should support the proposed clinical trial.

The exact antigen adjuvant combination that you would propose to use in the clinic should be evaluated in your preclinical study because we have seen instances where the antigen may contribute to the adverse reactions or the reactogenicity of the combination. The absolute cumulative dose in animals should be greater than the intended cumulative human dose. The annual study should include or use the route of administration intended for human use and should be given in episodic doses over several months rather than every day for two

weeks which might be more of the classical drug approach, so that the use in animals reflects how it will be used in humans.

The dose per injection should be equal to or exceed the human dose and the guidance that we've been giving sponsors is that if you've got your vaccine formulated in half a ml or one ml that you can give that to rabbits. Then you've got, on a per kilogram basis, you've got a considerable margin of safety.

And then there are controls to be considered. The adjuvant alone should be included, again as a control for the potential that the combination may be reactogenic and the antigen alone should be included or formulated with an aluminum compound to show that the adjuvant actually makes a contribution to the immune response.

Next one. I'd like to turn to the next couple of slides dealing with the vaccine clinical data. The safety, of course, is an essential aspect and we need to identify the potential specific adverse events that would be observed. Would be local and systemic reactions, as well as immediate and late and long term reactions and basically you

would be gathering the rates and incidents for the package insert.

For most vaccines, we're looking at being able to detect an event rate about 1 in 100. There should be an adequate safety data base. For example, for the newly approved acellular pertussis vaccines which are given to infants at two, four and six months, we have about 5,000 total subjects in each of those -- for each of those products. Of course, you have to keep in mind the risk benefit assessment that preventive vaccines are primarily for healthy individuals.

Efficacy is generally expected for all novel vaccines. It's important to define and come up with the prospective primary endpoint which will determine your sample size. Most vaccines, I would say, we're looking a target efficacy of about 70 percent in the per protocol cohort.

Immunogenicity and bioimmunogenicity for vaccines, most of the time we're talking about serologic antibody responses, is used -- we rely on immunogenicity quite a bit for determining manufacturing consistency. Most preventive vaccines include a clinical lot consistency study for approval.

The immunogenicity is used for bridging, for bridging from different populations, for bridging different regimens, dose and schedule, for bridging lots and also for bridging manufacturing changes.

Immunogenicity can be used potentially for the surrogate for efficacy if correlates of protection have been established. And the types of immunogenicity data we're looking at in terms of how a product would be approved, currently we're looking at percent responders or response rates, geometric mean titers or geometric mean concentrations of antibody and the reverse cumulative distribution curves which are very helpful. Those plot the percent of subjects that respond with increasing titers or concentrations.

But in addition to the clinical data that needs to be developed during the IND phase, there are also some nonclinical issues that definitely have to be considered. Again, consistency of manufacturer is very important for vaccines. The process is very important and we consider in some cases that the process defines the product. Quality control testing for product release has to be developed and a potency -- potency

is a difficult concept -- it's difficult to define. It's not necessarily obvious what's going to be an appropriate potency assay and it takes considerable thought and consideration, but basically, you need an appropriate potency assay to be able to dispense a safe and effective dose.

Stability would use the -- also would oftentimes incorporate potency, would include real time data to support an expiration date. Also keep in mind during the IND phase, again having appropriate immune assays and to be able to appropriately diagnose the disease that you are hoping to prevent.

And now I'd just like to spend the last few slides just going over some of the common pitfalls that we've seen in vaccine IND submissions. These could be the basis for clinical hold if they're serious enough because you have to remember the population for most vaccines is normal, healthy subjects.

In terms of preclinical data, sometimes we seen immunogenicity data is lacking and even if it's there the experimental data details are incomplete. Basically, we need information on the

lot, the dose, the route and the assays that are being used to evaluate the immune response.

Again, the preclinical studies are intended to support the safety and the dose proposed for use in the clinical study.

Next. Manufacturing information and variable conditions are frequent problems. I've seen folks say well, we centrifuge from 2 to 24 hours, but what's important is to include the exact information and the exact procedure that has been used to prepare the lot that you intend to use in your clinical trial. Again, lot, important lot release or in-process test results may be lacking and again this should be lot specific.

Potentially toxic substances, for example, organic solvents, validation of removal or assays for residual components should be included and adventitious agents, inadequate testing or inadequate information on source materials can be a problem for IND submissions.

Next one. Lot information is, of course, required. Sometimes we see in the protocol the lot that the sponsor plans to use has not been clearly identified and our recommendation is that you number your lots very early in development so

that you can follow each, follow changes in manufacturing and testing and relate that information to -- back one. I'm sorry -- to be able the relate the information obtained with various lots. It's important to identify the lots and number them early.

A summary table for lot information should be provided which would include the test that's being performed, the stage of manufacturer, what the acceptance criteria are and the test result and then the appropriate data can be attached.

And then there are protocol issues. We recommend that subjects submit the subject diary and/or the case report form to demonstrate how adverse events are going to be monitored. Assays describing how the immune response is going to be evaluated should be included. Again, the endpoints are critical as well as the case definition for efficacy studies and include the statistical analyses, including any planned interim analysis. And oftentimes we see inconsistencies in the submission. It's important to be consistent, that the protocol, the investigator's brochure and the consent form essentially detail the same study.

That concludes my slides. Just to quickly summarize again, preclinical studies should be appropriate to justify the clinical study, i.e., that the sponsor has concluded that it is reasonably safe to conduct the proposed clinical investigation, and the IND process is a mechanism to collect data to support the eventual license application. You need to have clinical safety and efficacy data and remember to keep in mind the product development so that you can prepare, come up with a consistent product at a safe and effective dose.

Thank you.

(Applause.)

DR. KEEGAN: Okay, our final speaker this session is Dr. Allen Albright. He's going to be talking about lessons from preventive infectious disease vaccines in relation to viral vaccines and adjuvants.

DR. ALBRIGHT: I might step around here so I can see my own slides. I'm here to talk about lessons that we've learned from preventive vaccines for infectious diseases, mainly viral vaccines and I feel like a fish -- I'm sorry, you can't hear me.

I'm Allen Albright. I work in the Division of Vaccines and Related Product

Applications Branch which is part of the Office of Vaccines at CBER. Again, these are preventive vaccines that I'm going to be talking about, but there is some overlap in terms of the way viral vaccines are produced, so hopefully this information will be helpful and relevant to you.

To give you an idea of where I want to go with the talk, first of all an overview. I want to touch on first of all the regulatory authority that we have for viral vaccines, give some examples of viral vaccines. We'll spend a great deal of time, not too much time, on safe viral vaccine production, mainly covering issues of cell substrate, viral seeds as well as product testing. I will touch a little bit on potency as well as consistency of manufacture. Again, these points have been discussed, but I will underscore those points and then talk a little bit about what we see in terms of IND pitfalls for viral vaccines.

In terms of the regulation of viral vaccines, again as biological products, these come from the Code of Federal Regulations, or the CFR, mainly 21 CFR 312 and 610 and these are to insure product safety. CBER also has Points to Consider documents and other guidance documents which are

very handy and that we use also to help regulate these products.

Other documents that we refer to and also use as guidelines would be the ICH guidelines as well as the WHO, World Health Organization standards or requirements.

I thought I'd put a slide or two in here about regulatory philosophy at CBER and again primarily is to evaluate and identify safety concerns as they relate to product manufacture and clinical design which Dr. Keegan highlighted earlier. We're also very concerned about quality control of your production process. Again, good quality control usually means a safe product and a consistent product, so that's important.

Other safety evaluation considerations and I put this up here mainly because I'm in the Office of Vaccines and I know most of the people here are concerned with Office of Therapeutics, but again there are differences in terms of safety evaluations, of course, for intended use, either prophylaxis versus therapy, again different risk benefit concerns; different target populations, are you going into healthy infants versus sick patients; routes of administration, cumulative number of

doses; severity of disease and whether the medical need is unmet, again, just evaluation for safety considerations.

Safety considerations as it pertains to product, again, there can be different levels of product purification or viral clearance. When we're talking about a live viral vaccine, again, we're basically talking about a filtered culture supernatant versus a recombinant subunit vaccine which could be highly purified and you have significant purification procedures, so there are differences in the level of purity.

The extent of inactivation, again, with a live vaccine there's no inactivation there. Inactivated, of course, would be inactivated and again would probably reduce the level of risk.

What examples do we have of viral vaccines which are licensed and again, these are categories of viral vaccines here on the left. They're live attenuated, such as the MMR, measles, mumps, rubella; the live oral polio and the newly licensed rotovirus vaccine. There are inactivated viral vaccines such hepatitis A, influenza, inactivated polio and rabies.

There are purified protein subunits which are made out of recombinant cell substrates or yeast such as the hepatitis B vaccine or peptides. Two other viral vaccine types are the viral vector recombinant, again, and the DNA vaccines. Again, both of these vaccine types of investigational at these stages.

I'll spend the majority of my time talking about the traditional viral vaccine manufacturing approach, but again, because viruses are produced in cell lines, some of these issues are cross cutting.

Okay, in terms of safe, viral vaccine production, we use a complementary approach and what I mean by that is there is characterization of a cell substrate for identity, endogenous and adventitious agents. We have certification of cell culture media, viral C history and characterization, your validation of manufacturing process for removal and activation of viruses, release testing of bulk and final products.

Also, there's in-process testing for adventitious agents to see whether those are introduced.

In terms of cell substrates, again, each manufacturer must characterize a cell substrate,

banked and used in production. In terms of history and isolation of the bank, growth characterizations, karyology and tumor genicity, freedom from adventitious agents. And these pertain to master, working and end of production cells.

In terms of identity testing, we have morphologies, species of origin, cell passage number, copy number and physical state of expression construct and again, these would pertain to recombinant proteins made from cell substrates. We'd ask that you would characterize your expression system, in other words, give a handle on genetic stability and integrity if that's applicable.

Okay, the rest, there's sort of a list of adventitious agent tests that we use. Again, some of these have been mentioned and I've listed the CFR references there on the right, issues such as bacterial and fungal sterility, mycoplasma, spiroplasma in the case of insect cell substrate, mycoplasma testing for cultable and noncultable mycoplasma, mycobacteria testing these both in animals and in culture, looking for adventitious viruses both in vitro and in vivo techniques, again, looking for acute lytic viruses as well as latent

viruses such as retroviruses or other oncogenic viruses.

Okay, in terms of adventitious virus tests in vitro and again, a lot of these are spelled out in the guidance documents, I'll highlight at the end. These tests are performed on monolayers of at least three different cell types including same species, tissues of substrate, human diploid cells, monkey kidney cells. There are tests for hemoabsorbate, hemoabsorbing and hemagglutinating viruses and again, as mentioned earlier, testing of your raw materials such as fetal bovine serum and Trypsin for bovine and porcine paraviruses as outlined in 9 CFR. And with the fetal bovine serum again, we ask that you use sources that are confirmed BSC free or certified sources for bovine spongiform, encephalopathy agent.

There are in vivo tests for adventitious viruses in your cell substrates including adult encephaline mice, embryonated hen's eggs and when appropriate in vivo assays including guinea pigs, rabbits and/or monkeys.

Okay, if you're using a rodent cell substrate such as mouse, rat or hamster, you can do the MAP, RAP, HAP tests respectfully and looking for

antibody production when you inject these cells into these animals and you look for species specific viruses which may be present in your rodent cell substrate.

In addition, you're going to look for lymphocytic choriomeningitis virus or LCM. If you're using a human cell substrate and we may ask for tests for viruses such as Epstein-Barr virus, CMV, hepatitis B and C, maybe there are others. You can use in vitro techniques, sometimes to look for these such as PCR. We're also concerned about the tissue source and the donor medical history for these types of substrates. So those issues are very important.

Okay, other adventitious virus tests, retroviruses is an important category and usually retroviruses tests are done by transmission, electron microscopy or TEM. Reverse transcriptase assays are used, as well as infectivity assays. And if appropriate, depending again on your cell line, we may ask for papilloma virus tests, adenoviruses, HHV 6 and others which become known to us.

Okay, once your cell substrate is characterized again another level of safety again would be your viral seed testing. And again, this

would apply for live viral vaccines as well as inactivated vaccines, where you use viral seeds. And I've summarized here some of the testing that will be required for typical master and working viral seeds for adventitious agents. You do control cells where you look for observation, hemadsorption and identity. You do supernatant of control cells looking at inoculation on cell cultures, microplasma and sterility. Once you've titered your virus or your viral stock, you do a viral suspension test where you look at sterility, microplasma, cell culture testing, embryonated egg inoculation, animal inoculation, RT testing, titer and tuberculosis and identity. Again, a lot of tests, but you want to make sure that these viral seeds are safe as well as the cell substrate.

Okay, once the seeds in the cell substrates are characterized and qualified, we may look at your cell substrate for validation of viral elimination from your manufacturing and purification process. Again, the tests that I've described previously can detect adventitious agents, but your manufacturing process may serve and should serve to remove these types of agents as well. Again, this

would apply for inactivated viral vaccines and/or cell line-produced recombinant proteins.

This would involve -- I'll highlight these four here: selection of appropriate viruses in these types of tests, physical removal versus inactivation, kinetics and completeness of inactivation and an estimation of combined effect. I won't mention these two, but these are outlined in the Points to Consider document. Each step needs to be analyzed for its ability to eliminate virus as well as you can do a scaled down manufacturing system approach to look at that.

Okay, viruses used in viral clearance studies typically shall resemble viruses that could contaminate the product, should represent a wide range of physical chemical properties and include relevant viruses or specific model viruses and nonspecific model viruses.

What are model viruses? Again, these would be typically model viruses of DNA or RNA genomes, enveloped and nonenveloped, low to high physical chemical resistances, small to large and viruses that can be grown to high titer. In other words, viruses at different ends of the spectrum, but again, if you can eliminate the small and the

large, typically anything in between should be also removed, so that's the idea with that.

And distinguish between physical removal of these adventitious agent or viruses versus inactivation of the viruses in your manufacturing process. Both serve as a mechanism for viral reduction, but again, mechanism of a loss of viral infectivity should be determined at each step.

Okay, just a point here on effectiveness of inactivation. If you're inactivating your virus in cell culture, you need to validate the kinetics of the inactivation and that's where you test residual infectivity of samples during inactivation the process at different time points. The purposes of this is to establish an inactivation curve and we hope to see a linear decline in infectivity at time of inactivation.

Included in that, but separate, is also a test for completeness of inactivation, where once you've generated your material or your product, you want to test multiple dose equivalents such as two time points and typically this can come at the middle and the end of the inactivation period and what you're looking for here is that there would be no CPE or cytopathic effect or immunofluorescence

antibody results in cell culture indicative again of live virus.

Very quickly here after your viral inactivation is finished you want to do an estimation of combined effects of the inactivation and the clearance from your manufacturing process. And there's a need to quantitatively estimate the overall level of virus reduction achieved. It's important to demonstrate that in your process there's an excess capacity for viral clearance within your system. It's important to compare the amount of virus eliminated to the amount of virus present in the unprocessed bulk drug and that's usually done by TEM or transmissional electron microscopy.

Lastly here, it's important to calculate the virus reduction and estimate the virus particles per dose of your vaccine. That's outlined in the ICH guidance document referenced here. So how much did your process really eliminate virus compared to what you inactivated versus what's in your final dose.

Okay, so you characterize your cell substrates. You characterize your viral seeds. You've looked at your manufacturing process for its

ability to remove these agents. You want to do final or product testing as well as to finish your complementary approach. Again, purity tests and I've listed here a number of things that we look for, residual cellular protein, DNA, RNA, serum protein. You can use BSA as a marker. Endotoxin, moisture, ancillary products from your manufacturing process such as protease inhibitors, antibiotics. Again, the cellular protein, I'll just highlight, sometimes this is important in terms of what types of cell substrates you're using, in terms of what kind of cellular protein might be hanging around.

Sterility, of course, is done; general safety tests, except for those products which are exempt, specified products such as therapeutic DNA vaccines are exempt, are in that category.

There's also product release testing of bulk and final products. Again, we also like to look at any in-process testing that you may do to prove that there were no adventitious agents introduced during the manufacturing process.

Potency. I'll highlight that in a minute is done and then there may be other characterization tests that are not part of your lot release, but again such as mass spec, N and C-

terminal analyses and isoelectric point. Again, the more characterized your product is, hopefully the more safe it is. So these tests are important as well.

To touch on product potency again this has been highlighted before, but again potency is basically the specific ability or capacity of the product to affect a given result and ideally this should correlate with clinical activity in an ideal world, but we like to see quantitative in vitro and/or in vivo tests for potency in our office. And I would distinguish between biological activity versus expression for potency assays. And what do I mean by that? Well, animal assays typically, I know there's a lot of variability associated with animal assays. They give you a level of measure of biologic activity. For a vaccine, this would be an immune response, an antibody response. And typically we see assays in mice or guinea pigs. There are other assays such as ELISAs which people can use for potency, but these usually measure antigenic content, not necessarily biological function. However, and sometimes we like to see both, if sponsors can correlate an in vitro assay to an in vivo activity, we may accept an ELISA or an in

vitro assay, but that has to be correlated and validated before we'll accept that.

Potency lastly is important because it's usually associated with product stability which is important.

One slide here on consistency of product manufacturer. Again, we like to see that there's quality control of the production process to insure safe, consistent, stable product. Is there lot to lot consistency, etcetera.

One point here, specifically, I know we've been telling sponsors for live viral recombinant vaccines when you do a consistency of manufacturer proof of concept, I guess, a lot of times we just see Western blot data that's given and Western blot alone doesn't really give you a quantitative estimate of the proportion of virions expressing the recombinant product, although it's an assay that shows that product is being produced. We've been recommending that immunoplaque analyses of the viral stock be performed to show the proportion of production lots which are expressing your protein.

Again, this would demonstrate consistent expression of the protein as well as demonstrate that the gene is retained as a stable insert.

Preclinical, I will not spend any time really on this other than to say this is important for viral vaccines as well. It's important to show that data, in vivo data and in vitro data support the product's clinical use, namely, for toxicity activity and a possible efficacy. Again, as Drs. Chandler and Essayan mentioned, adjuvants, novel adjuvants like cytokines and other immunosimulatory molecules, we license adjuvants with the product, not as a separate entity, but for novel adjuvants such as these we usually require separate preclinical studies to be done.

Real quickly, IND pitfalls for viral vaccines again would fall under the same categories as Dr. Chandler just outlined, but in terms of all the testing that has to be done for viral vaccines, obviously,if there's insufficient information to assess the safety of the product such as removal or inactivation of these adventitious agents, that's going to be a problem for your IND submission or it's going to be a reason that raises question in our minds of safety. And usually the testing and

sometimes sponsors have done testing, they just don't provide the documentation to us or they haven't done it at all. So they can fall into different categories, but again, these would include tests on your master and working cell banks, master and working viral seeds and your bulk and final product tests.

Other issues of manufacturing, again, variable conditions described, how did you produce your viral vaccine? It's important to describe your procedure. In-process testing may be lacking, again, there may be failure to validate removal of potentially toxic substances from your manufacturing process.

So in summary, sponsors should make every effort to produce and I probably should have put these in quotation marks, should make every effort to produce quality products which are as safe as possible. Quality, per se, cannot be tested into the product, however, appropriate testing of cell substrates, viral seeds, bulk and final products, as well as in-process testing can help insure the safety of these vaccines.

Thank you.

(Applause.)

DR. KEEGAN: Okay, since we're running a little bit short of time, I think what we're going to do instead of having a question and answer period is let you go to lunch and we will start promptly at 1 o'clock because we have to be out of this auditorium at the designated time here, so we need to get going. If you have questions on regulatory principles that were presented, feel free to catch any of us and ask us questions.

DR. PURI: The cafeterias, as I indicated, there are two of them in this building, one in the first B-1 level and the other one is on the second floor. There are other cafeterias that are available, please pick up the map from the Registration Desk. So we'll see you at 1 o'clock.

(Whereupon, at 12:14 p.m., the workshop was recessed, to reconvene at 1:00 p.m., Thursday, December 10, 1998.

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

(1:01 P.M.)

DR. STEINMAN: We have a busy afternoon and it's time to hear of a number of different presentations on the clinical use of dendritic cells. The plan is most of the speakers really don't have a lot of time, so there probably won't be much time for discussion in association with each talk, but we do have a panel discussion at the end and we'll try to have all the questions and discussions come up then.

Okay, so it's 1 o'clock and our first speaker is Gerald Marti and it's on the current clinical use of dendritic cells, points to consider.

DR. MARTI: If I could have the first slide, please? I thought I would begin my discussion this afternoon with a partial list of the so-called cellular or somatic and genetic therapies that I have seen come across my desk or this division in the past ten years. Basically, this started with the LAK and TIL cells and then moved into transduced cells and a lot of subfractionation enrichment procedures for cells which brings us today to dendritic cells.

I would say that during most of that time I wanted to point out that flow cytometry was a very useful took and remains a very useful tool in studying those. In the last two years, there have been 40 submissions to the Agency that involved the title "dendritic cells." Two of those involved HIV, three were hematological malignancies consisting of multi-myeloma, amyloidosis and chronic myelogenous leukemia. There were 14 involving primarily metastatic melanoma and then the remaining bulk involves solid tumors.

The cell preparations were varied. Some used whole blood, some used unmobilized aphoresis products. Some used mobilized apheresis. Those who mobilized used either G-CSF, GM-CSF or flt3L. Almost all of these with the exception of those individuals are investigators who used whole blood, used an enrichment procedure and most often the enrichment procedure was buoyant density centrifugation.

The cell culture and I'm -- since the majority of these types of planned therapies were in the autologous setting, the goal was to obtain autologous peripheral blood mononuclear cells to derive the dendritic cells and in the vast majority

GM-CSF and IL-4 was used to derive or expand the dendritic cells.

In some situations, interferon gamma was used and in select situations, the autologous PBM cells were either purified to the level of CD8 cells or various procedures to enrich CD34 cells and then the typical cytokines of GM-CSF and IL4.

In some situations, activation was accomplished by using OKT3. That can be soluble or on beads and tumor necrosis factor alpha and flt3L was also used.

The antigen preparations, in all honesty, I attempted to summarize the antigen preparations, but if I started in the upper lefthand of the corner and wrote down to the right hand I would not have -- I would not be able to tell you. There is such diversity in antigens that are used that I think it is crying out for some kind of consistency. Dr. Raj Puri had a very long list of the various and sundry antigen preparations that have been used. This is representative.

In addition, the antigens that are being used in these cultures, some individuals use the adjuvant, the incomplete froins adjuvant. some individuals are using KLH and some are using tetanus

toxoid and some are using KLH and tetanus toxoid together.

The culturing and antigen pulse is also somewhat variable. Sometimes the culture and the pulsing is overnight. Sometimes it's 30 to 48 hours. Sometimes it's 6 to 7 days with the last 24 hours or overnight being the antigen pulse. Usually, it is followed by some minimal wash and it's used immediately,although some investigators are using cryopreservation for either delayed juice or serial injections. One thing that is not always so clear is exactly what the cell dose is or the target dose is. And the roots of administration, although they tend to be primarily IV, some investigators use subcut., some combined IV and subcut. and I left the KLH and tetanus toxoid on there because I wasn't sure as to what level they remained in the product that was being infused.

Also in this modern era of the cytokines, they are now being used as adjuvants and some of the adjuvants that are being used are being administered at the same time as the cellular vaccine is IL-2,
G-CSF, GM-CSF and interferon.

Okay, cell product characterization, morphology. I appreciated seeing Dr. Steinman's morphological presentation this morning. I'm not suggesting that morphology become some kind of gold standard, but I do think it would be useful for investigators to make a slide once in a while and look at it, if for no other reason to do a gram stain.

(Laughter.)

I also have a prejudice that most immunologists have never seen a lymphocyte --

(Laughter.)

-- let alone a dendritic cell. The whole area of tumor specific assays, be they proliferation cytotoxicity or cytokine release assay is going to be the subject of the next session or the third session tomorrow. In the remaining time that I have, I want to share with you our experience in flow cytometry although it does not relate directly to dendritic cells, we believe that it's applicable.

The common immunophenotype, Dr. Steinman outlined very nicely the problems this morning. You have the origin of the dendritic cell. Is it a Langerhans cell? Is it myloid? It is monocytic?

It is a dendritic cell? I recently learned that lymph nodes now are believed to have two types of dendritic cells. And is it an immature form or is it a mature form. This is a rather early phenotype suggested by Peters, et al., CD-1, CD-14 and CD-33, more or less marking the myelomonocytic with the co-stimulatory molecules, CD-40, CD-64 and some adhesion molecules.

And of course, the classic bright expression of Class 2 antigens here listed as HLA-DR, DP and DQ.

In a more recent article, Banchereau and Steinman have indicated that while many of the monoclonal antibodies are not specific, they are nonetheless very useful in identifying dendritic cells. Two of these antigens were mentioned this morning. Or one of the reagents was CD-83 which recognizes an immunoglobulin superfamily member and P-55 which is an actin binding protein. We learned this morning that the function of that is not known. The reason it's called an actin binding protein is on the basis of its homology.

Some of the things that I think people should start directing their attention to is the absolute count in whole blood and I will come to

that at the end of my talk, also, yields in apheresis, what's happening to these markers during culture and the final product. There's some concern here because in the final product we see values like in 1 to 5 percent dendritic cell cells and other investigators say that they have 30 to 40 percent. That's a tremendous amount of variation.

Some of the sources of variation, although this was from an earlier study that we did, I think that these are universal. A lot of flow cytometer operators don't have a unified instrument set up. They don't know what you mean when you say a calibration curve. And compensation controls, I will tell you, that in the beginning we were quite cavalier about those and then believed that we became very intellectual and sophisticated about them. Yesterday afternoon in a two-hour session with Carlton Stewart, I realized that all of our ideas concerning controls for color compensation have been wrong.

Saturation staining is often not accomplished and when you're looking for rare numbers of cells, I think you need to consider the so-called Lyse and No Wash procedure. If you're looking at

CD-34 cells in an unmobilized preparation where there's 1 to 5 per microliter and you do one centrifugation and lose half or all, I think you would agree that a Lyse No Wash procedure is useful.

I can't stress the need to evaluate and not only methods of Lyses, but fixation and not all antigens are the same. Some further considerations. Two and three color is probably a minimum. No regulatory body recommends single parameter or a single color flow cytometry any more. It has no power for resolution. I think that it would be very useful if a consensus panel of reagents for blood dendritic cells can be defined. I may come back to that in the panel session and also a consensus protocol and a local protocol, so that you can have side by side comparison. Otherwise, you will have two laboratories in the same institute doing an analysis on the same sample and getting two different answers and they will say that the other lab is wrong and they're right and go on their way. You have to do a side by side comparison. But even that is not without its problems. Collect adequate number of the events. I think that depending -- if you're down at the 1 in 5 percent level, I would suggest that you start collecting 50,000 events and

not just 5,000 and 10,000 events that are used so commonly.

Fluorochrome configuration, it's very important. What fluorochrome do you put on the monoclonal antibody and what combination of reagents do you put together? And gating strategy I will touch upon in just a moment and I will also in a subsequent slide mention quantitative flow cytometry and training, I won't elaborate on.

Beads. Use them. There are six manufacturers. Choose one and use them. It will give you a calibration curve. Pick a set of beads where the cells that you're looking at, the fluorescence intensity falls between two of the beads and then you can extrapolate off of your calibration curve.

Gating strategy. Basically, this is a lineage negative approach. Label everything in there that you're interested in and put the double negatives, the basophils and the dendritics down in the third quadrant or the double negative quadrant. Recently, there's a monoclonal antibody that's been identified, ILT3 that appears to be specific for dendritic cells in that particular scatter gate. Also, more recently in addition to CD83, a

monoclonal antibody labeled DC-LAMP, a lysosome associated membrane glycoprotein which was mentioned this morning, has also been reported.

The reason I listed that is that is a cytoplasmic, an intercellular antigen and that has a little more stringent requirements in flow cytometry than surface markers.

If you do this approach where you essentially identify the populations there using that cocktail approach which was actually first used in looking at stem cells in the mouse and then later applied to stem cells in the human, you see that in that double negative quadrant there's a group of cells and if you come over here to the -- I guess I have a pointer here, well, I'm shooting myself.

(Laughter.)

Anyway, here's an isotype control. Here is the ILT3 isolating the dendritic cells separate from the CD34 cells. You can also isolate them using -- see them using CD34 in HLADR and when you put the HLADR and the ITL3 together, you get co-expression.

Now if you take that same approach and add a third antibody in a cocktail on this axis, you are able to identify the basophils and the CD34 stem

cells hematopoietic progenitor cells and you have nice isolation of the dendritic cells.

I want to point this out to you that this is a sample of whole blood and when I started reading this material, one would have thought that dendritic cells were a real rare event in unmobilized blood. At least in this study it would appear that about four tenths of a percent of dendritic cell is somewhere in between the number of resting or unmobilized stem cells and basophils. That should make the task a little bit easier, but this data needs to be confirmed.

I am going to close on just to bring to your attention two reports. This was an NIH consensus report concerning flow cytometry and there's a subcommittee report in that that involves standardization. Finally, just recently there is an issue of Cytometry, the entire issue is a special issue dealing with the emerging consensus of quantitative fluorescence and that was in October of this year. And on that note, I'll end.

Thank you.

(Applause.)

DR. STEINMAN: So again because of time constraints, we're going to move on and we'll have the questions and discussions at the end.

Now you've heard a lot of new terms so far, but I wonder if you thought about what you call a workshop that has 500 participants. It's called a Texas Workshop.

(Laughter.)

Our next speaker is Jacques Banchereau from Texas.

DR. BANCHEREAU: Good afternoon, everybody. I'd like to thank Raj Puri and the organizers for inviting me to talk with you about the dendritic cellite. As you can see here, for those who have never seen dendritic cells, that's how they look, although we have changed a little bit the color recently, thanks to our confocal.

As Raj told us, the dendritic cells are present in the major form in the tissues and they're derived from nonproliferating precursors that circulate in the blood, that about 4 percent cells that we just heard about circulate in the blood and the cell originating from the proliferating progenitor in the bone marrow.

Now whenever a problem arises, a virus, a bacterium or whatever, the dendritic cell's job is to capture those antigens and then to transform them into the secondary lymphoid organ where they will present the antigen to the specific lymphocytes. I mean during that time, the dendritic cells may turn into this major form, then permits the activation proliferation of the T-cells and also the B cells eventually die by apoptosis and the T-cells that have multiplied are now going back into the periphery and can go back to the site of injury because of the expression of various addition molecules on those inflamed endoterial cells.

Now the T-cells can directly act on the cells which have been injured or it may be an indirect activation of either using the feline K cells. Now I'm going to show you some slides which represent, sort of a view of dendritic cells which are different from the ones you might have seen. Dendritic cells are sitting here in tissues like those flamingos in Lake Kibazu and whenever the antigen or the pathogen come in, the dendritic cells just fly out of the tissue, carrying the antigen. They are lending in the secondary lymphoid organ and

there they do their job which is to select the rare antigen specific T-cells here, a zebra --

(Laughter.)

-- in the middle of the nonspecific T-cell of the new. And now the job of the dendritic cells is to allow the activation of that zebra and its suspension into the head of the zebra.

(Laughter.)

Now they will now be able to really do the job. The final job of the dendritic cells is to allow the differentiation of the zebra into cytotoxic effect on as this series of crocodiles.

(Laughter.)

So basically when you want to do dendritic cell therapy you've got to get the flamingos and get the flamingos to identify the zebras and get the zebras to become a crocodile and that's basically all what we are trying to do.

(Laughter.)

The problem that we have had with dendritic cells is we know there was a homogenous population of pink birds, but it is not. There is a considerable heterogeneity between the white birds, the pink birds and the deep red birds. And this is the heterogeneity which is in the label of the

maturation level from a precursor to an immature to a mature dendritic cell. This is the heterogeneity in terms of the various subsets of dendritic cells. And up until a few weeks ago I would say I have seen in the blood three different precursors of dendritic cells. I would be convinced that the monocytes would be precursors of dendritic cells as well as macrophages, that there is a population of CD11C plus cells that Raj had identified that we had identified and others have identified which we felt were the germinal center of dendritic cell precursors and that there was also a population of CD11C minus dendritic cell precursors which we had identified in my former operation in France as being a plasmacytoid T-cell, very interesting population of dendritic cells.

I do believe now that there's a little less because of the specific experiment. We have sorted the CD11C plus dendritic cell precursors from the blood and have found that whenever we grow those cells with GM and TNF, they become dendritic cells while if we grow them with M-CSF they become macrophages.

Now furthermore, you see that when they have been grown with GM and TNF they are very strong

activator of allogeneic CD14 sensors where I've told you the proponent assay for all our testing, while the macrophage would be for activators. So we do believe today that this is a kind of an activated or different shaded monocyte that has that dual potential. We believe, actually, that a monocyte that is cultured with R4 is basically that cell and possibly in the circulation that cell may be a monocyte that has encountered L4 possibly from mass cells or others.

Just one words about the CD11C minus dendritic cell precursor. We believe this cell is really the human equivalent to the mouse lymphoid dendritic cell. We believe it's for one reason is that Hurgenspitz has identified in the blood of -- in the human blood a precursor of T-cells which actually has the characteristics of those dendritic cells. Furthermore, Hurgenspitz has also demonstrated that dose plasmocytic T-cells, the CD11C minus dendritic cell express the pre-T-cell receptor.

So we just know very little about, except for the very important finding of Joung Jin Liu now with Denex that doses may be important inducing TH2 responses.

In vitro work has certainly sparkled a lot of enthusiasm for dendritic cells and their use in therapy. Now really two major pathways, as you know, the pathways that we've been involved in identifying from the CD34 progenitors when Christophe Coe was a student, in France and the pathway that Ralph Steinman and Antonio Lanzo-Vecchia have popularized from the blood monocytes.

I'm going to talk mostly about that because there will be more talks about this during this day. While we have been focusing on the assay and telling you that there are actually two subsets originated from CD34, subsets which is a typical Langerhans cell subsets which are the dendritic cells in the epithelial and the subset which we like to call the interstitial DC subset which is in most of the other tissues.

That came, really, in experiments done by Christophe almost 10 years ago now when he identified that TNF alpha was synergizing with either IL3 or

GM-CSF to induce strong proliferation of C34 progenitor and that was at that time totally unusual, so Christophe had grown the CD34 from the cow blood with L3 and you have good expansion here

after 8 days or with GM-CSF here after 20 days. But addition of TNF was resulting in a considerable expansion. I insist on that slide, not only because it took us into Texas, so to speak, but also because it is showing a considerable expansion. The problems that we have met with the human blood, CD34 by comparison to the cow blood. And although also those experiments we have done with fetal calf serum and what I will report afterwards is not done with fetal calf serum.

So when Christophe looked at those cells, the C34 cells neither expressed CD14 nor CD1A, but after Day 3 or Day 5 we had two populations clearly showing up, a CD14 plus and a CD1A plus and then when the days pass by, Day 7, 8, we start to see another population in between and at the end we only have CD1A cells.

Now Christophe has done all the studies and demonstrated that indeed the cells with the CD1A are true Langerhans cells because they express Birbeck granule, because they express the lag antigen which is associated to Birbeck granule.

On the other side, the cells which have the CD14 and which even become CD1A positive are the other dendritic cell population and myloid

interstitial DC which expresses CD68 and something typical of the dermal dendritic cells which is factor 13A of coagulation. So we can isolate two different subsets within this population and it was for us an attraction to do the clinical studies with those cells for that very reason.

We found with a lot of effort, we found one major difference between those two populations in addition to those phenotypic differences. It was an assay relying on studying the B cells. Here in this menage a trois where the dendritic cells, the T-cells, and the B cells permit very efficient immuno response, Christophe found that the Langerhans cells of the interstitial dendritic cells were both about to induce and enhance the B cell proliferation, both about to induce the differentiation of memory B cells into plasma cells, but only the CD14 population, interstitial dendritic cell population was able to induce what we call the primary B cell reaction. That means to take a naïve B cells and make it secrete IGM in a response to L2 and this is why we are very interested or so in testing in vivo whether there will be considerable differences between the dendritic cell population.

The Langerhans doesn't do the job at all, while this population does the job.

Now that brings us with -- although we start to have some idea about the population, we haven't solved all the problems. We do believe that the monocytes and the CD14 population really correspond to the dermo dendritic cell and interstitial dendritic cell population. Now where the Langerhans cells come within that whole scheme is complex to explain at the moment, but in view of the research of Frederick Geissman who said that with TGF beta I could get monocytes to become Langerhans cells, something I know is being discussed by other groups.

So that is a question that is not entirely clarified, at least in my mind. If you have a solution, help me, please.

Of course, doing in vitro studies is fine, but to really prove the point, you really need to do the in vivo studies and it was very difficult for me to be in the hospital nearby, the research center in Lyons. So we decided in a positive way, we found a hospital and built a research center to do those experiments and this is why I moved to Baylor at Dallas where we want to study the role of

dendritic cells and induction of immunity in cancer and in infectious disease and we will discuss that next time.

To do that, we have several possibilities, of course, which is the source of the DC precursor/progenitor, the CD34 HPC is, of course, one which we developed and can talk a bit about the monocytes. There is the FH3L APC and we're going to hear today about this puzzling cell, the fibrocytes. The problem also with CD34 HPC is immense. It's not so simple because according to the way you mobilize the C34 HPC in the blood, either you take it from the bone marrow, or mobilize with GCSF, GM and FH3L, we will see differences.

The problem of the isolation of the progenitor and precursors, what methods, what technique, purified, not purified. This is going to be important. It adds cost, but it also adds bias in the population that we're isolating.

The problem that we're going to have to generate the dendritic cells, ex vivo, we will hear about making dendritic cells without cytokines. I have not been able to do it and the problem of putting the cytokine is a problem for, of course, numerous reasons. We need to worry about the

quality of the DC and what are the criteria that we use for releasing those cytokines.

Well, manipulating dendritic cells can be done at three levels and today I'm going to be insisting on one which is the closest to the clinic, is this one where we just manipulated them ex vivo. We load them. And I want to discuss in great length the loading of the dendritic cells and then the reinjection.

We are now studying dendritic cells generated by recirculating with GCSF. We are isolating the CD34 HPC and we grow them with GM-CSF and TNF. We started with the stem cell factor. We are now doing experiments with flt3L since we didn't see that many differences.

Today, actually, our first patient is showing up to get his GCSF treatment and Monday he is going to get his aphoresis. We're going to purify the CD34 cells on Tuesday. The CD34 cells are going to be frozen in aliquots. What we're going to do from Tuesday, we're going to ex vivo generate the dendritic cells and first we will do a launch. We will see whether those cells can be giving dendritic cells. It's going to take about two weeks. Once we are happy, we're really going to

do the real experiments, throw the CD34 cells, grow them and then load them. We are using KLH in our experiments only in the fourth injection are we using TT because of Ruff and Garontula experience that this may really give fever if we are to put too much of it. And then we are loading with the four peptide of a MART GP100 tyrosinase MASH 3 which is also a big problem is the loading of the peptide, how do you load with the peptide and so forth. We have been discussing that in great lengths with Myos Null. We're going to try something. We hope it's the best.

Now those dendritic cells are administered in form of injection into a patient every second week and the one question we have decided being unable to address what is the best, we say we're going to use part IV and part subcut or intradermally probably and this is going to be in three different sites. The dendritic cells that we generate actually are dendritic cells at an intermediate stage of differentiation. We don't want fully immature. We don't want fully mature. We want in between. We want dendritic cells as those that have encountered the pathogen that will migrate optimally to the lymph node. The numbers

that we're injecting are between 10 to 5 and 10 to 6 DC per kilo for 70 kilo individual. No comment on that. It's between 7 million and 70 million, four times. And one month after the end of the trial of the dendritic cell injection, we will treat the patient with interferon alpha because it's an approved therapy of melanoma and more important to us, because if we generate the crocodiles, we want the crocodiles to recognize the antigen which is the MHC Class 1 and we hope that the MHC Class 1 will be off regulated by interferon alpha on those cells.

Now a lot of issues. When we have used and when we are using for our cells, the criteria of cell expansion, the criteria of the percentage of the CD1A and CD14 and the ability to induce T-cell responses. Of course, using those criteria, we had to study all the parameters of the purification serum or the nonserum, the length of the culture, the stages and so forth.

The cultures are presently done in a serum-free condition where we have an expansion which is between 1.9 and 3.4, expansion that is reaching a maximum after 10 days. We start cells at $5x10^5$ per mil and we expend about three fourths. The third that we generate contain both CV14 and

CV1A population as we have seen with the cow blood, in spite of the absence of serum. Now the percentage of positive cells is variable. We have between 10 and 30 percent of CD1A cells. We have between 25 and 40 percent of CD14 cells. All the other cells are Class 2 positive, are DR positive and we believe they represent some kind of undifferentiated monocyte precursors or whatever, something like that. Those cells reach a maximum after 9 days of culturing usually. Now if we add autologous plasma to the culture, we don't get an increase of both cells usually. It doesn't help us. In both cell types can induce a neural reaction, be that's totally serum-free or be that in autologous plasma. The one consideration we are doing now is we are testing, we will be testing for each of our patients there on plasma because they have been mobilized. At the moment we haven't been able to do that specifically.

So for releasing the dendritic cell, we just want to have cells which are more than 80 percent viable. Two hours before administration we do Giemsa staining and they don't look like flamingos. The phenotype we do study two hours before administration Class 2, CD86, CD1A, CD14. We

have a gram staining 24 hours before administration, a bacteria culture 48 hours before administration and then every region is tested for endotoxin content.

Now this is a trial that we are doing with the peptide. And for us it's kind of a setting of the scheme. Many alternatives can be used to load the dendritic cells. We will hear about loading with RNA from chem. We will possibly hear about recommended polypeptide. We will hear about viruses to transduce the DC. We are very interested by a finding of Nina Bhardwaj with the capture of apoptotic body and I will summarize a bit what we're doing now and the question because we are considering our first trial in this context.

Monocyte-derived DC can capture apoptotic body, about 25 to 30 percent of the cells will capture apoptotic body as measured by 7AD ADA stainings. 7AD is stains DNA. Here you see with confocal microscopy for section of the monocyte-derived dendritic cells one hour after the capture. You see here the first two compartments. You don't see the tumor body. Here in the next section you see the tumor apoptotic body in red because it is a red stain and then you see the Class 2 which are not

fused. If you leave four hours of incubation, you see the fusing of the apoptotic body and the Class 2 compartments possibly along for the loading of the Class 2 and Class 1 antigen. Actually, to demonstrate that it is very simple to take the monocytes, make them the DC image, show the DC as Ralph told us, and then load the DCs with EBV-LCL prostate cancer, melanoma or breast cancer apoptotic bodies and then test with the T-cells from this individual will proliferate in response to the antigen.

Dendritic cells is loaded with EBV-LCL, a low EBV-LCL, can induce the proliferation of CD8 T-cells provided we see fully ligan activated DC and IL-2. So we have a CD8 proliferation here while macrophage, as Ralph told us earlier, are not able to induce the proliferation of those CD8 T-cells.

Now should we use necrotic or apoptotic body? That is really in some way a very semantic issue and we can discuss that later, but the way we induce apoptosis results in capture which is not as efficient as the capture of necrotic body yet with the apoptotic body, we get very good T-cells, CD4CL response. We don't get that with a necrotic body.

Now this in that case we have loaded with prostate tumor cell lines and we get a strong proliferation of the T-cells which are autologous to the dendritic cells.

The purification of those dendritic cells is going to be a problem. Here the monocytes are enriched by adherence. Here, they're enriched by depletion. When we do depletion, we get better dendritic cells or purer dendritic cells, all of them being CD1A positive, while we do adherence, we don't get them all to be CD1A. So it's going to be a question, of course, this is more expensive.

The problem of the tumor cells as Raj Puri told us is a big problem, autologous tumor tissue, allogeneic tumor tissue, what is the criteria we need to use for those tumor cell lines, what is the matrix for generating those tumor cell bodies from apoptotic body, necrotic body, should we fractionate? What is the quality of those bodies, the composition, all those are going to be extremely important questions that this small audience of 500 people will certainly address.

And the way to load the body. That brings us to a possibility of manipulating directly the product, the crocodiles. I mean we can how from

the breast cancer patient receive heavy chemotherapy, generate the crocodiles in vitro whether they generate them in vivo and regenerate the crocodile to the cancer patient and providing the cancer patient with her chemotherapy which otherwise would kill all the T-cells.

There's finally another way of manipulating the dendritic cell system is directly in vivo with the DC mobilization. And so the Immunex product, the fH3L is an extremely important molecule in that respect. Now I just want to show you one slide which is the monocyte from lymphoma patient before fH3L and the monocytes after fH3L. So not only do we increase monocytes, not only do we increase by 5 to 50 fold the DC precursors as shown by the Immunex group, but also we get totally activated cells which may be dramatic antigen presenting cells.

And very importantly, in a study done on two of my former patients, these T-cells are in vitro energetic while after two cycles of fH3L, the T-cells of the patient now respond in vitro to the tetanus toxoid which can bring us a lot of question.

When the work was done in this new dream team at Baylor with particularly I'd like to mention Carolina Palucka and Maya Nouri-Shiraz for their work with the apoptotic body, the work on confocal was made by Jean Davou, we have done with the work on Fed. 3 with the group of Immunex with Charlie Marichevsky and Daniel Caron. Clinical trials are led by Joe Fay. The clinical trial in the prostate cancer will be led by urologist Mike Goldstein and the work is being done in Baylor Institute and Center of Immunology Research and thank you for your attention.

(Applause.)

DR. STEINMAN: Well, that was great, Jacques. We'll again save the questions for the end of the afternoon. You must stay.

So we'll move on to David Urdal from Dendreon who will talk about clinical trials and product development with dendritic cells.

DR. URDAL: Thank you, Ralph. I'd like to thank the organizers for the kind invitation to come and speak this afternoon and describe to you some of the work that we're doing in developing immunotherapies around the use of antigen pulse dendritic cells. I thought what I'd do this

afternoon is organize my talk in the following way, that I'll give a very brief description of the biology of the dendritic cell. We've already had a lovely introduction to that by both Ralph this morning and Jacques just before me. Jacques is almost an impossible act to follow. I have no zebras or other animals on my slides.

I'll spend most of my time talking about the Dendreon process and what we're doing to really distill this type of a process into one that we can actually take through the clinic and hopefully some day into the market place and then very briefly at the end talk about some of the applications that we're making of this process to the treatment of cancer and some of the status of where those studies are.

As we've heard, dendritic cells are the most potent antigen presenting cells in the immune system. They play a central role at conducting the initiation of recognition of antigen by T helper and cytolytic T-cells by their capacity to process antigen and present that antigen in the context of MHC Class 2 or Class 1 molecules, leading to ultimately the creation of effector cells that have

the capacity to eliminate virally infected or tumor cells.

Not just the MHC, T-cell receptor interaction that mediates this, of course, but there's a constellation of co-stimulatory markers that make dendritic cells the potent antigen presenting cells that they are. This is just a subset of the ones we've already heard a great deal about, including

CD-80, 86, IKM1 or CD54 and LFA3.

We've also heard of a number of ways that antigens can be presented to dendritic cells and if I can draw your attention to the bottom of this slide, clearly, the forms of antigen that can be used include whole protein, DNA or RNA and coding the protein antigen or synthetic peptides as well as we've heard, tumor lysates as well as whole tumor cells that would be able to interact with dendritic cells in presenting their antigens to those cells.

Clearly, depending on the chemical form of the antigen that you pick will determine to some extent the dendritic cell that you may wish to look at in the clinical situation. For example, peptides that you might want to exogenously load on to dendritic cells, you might choose a mature dendritic

cell that has its mature and high intensity display of MHC Class 1 or 2 molecules on the surface. In contrast, if you're working with protein or DNA which would require uptake by dendritic cells and processing of the antigens into the epitopes that can be expressed on MHC Class 1 and 2 it would be driven to work with cells at an earlier stage of dendritic cell differentiation.

As we've heard, the dendritic cell is derived ultimately from the hematopoietic progenitor cell, the effector cells that we're talking about in most of the studies this afternoon are either derived from that cell ex vivo with the appropriate cocktail of cytokines provided or for monocytes, again with the appropriate cocktail of cytokines. And as I'll describe we're working with cells that we isolate directly from the peripheral block to derive potent antigen presenting cells that we take into the clinic.

Having their origin from the hematopoietic progenitor cell, the dendritic cell clearly originates in the bone marrow. It travels through the peripheral blood to take up its residence in tissues where under its natural biology it would come in contact with antigen, find its way

to lymph nodes and see the high flow through of naive T-cells from which it can pick the zebras that Jacques alluded to.

Clearly, what we're doing then in a clinical and a commercial setting is finding a way by which we can isolate dendritic cell precursors from some source and in our case we're looking at a blood collection to take those dendritic cell precursors, present antigen and induce their maturation in vitro by some fashion and then having created the antigen loaded dendritic cell, reinfuse that cell back into the patient.

And of course what we're looking at then is the process whereby the patient is coming into the clinic. Chances are they will look at pheresis as being performed at an axillary site. The white blood cells are being delivered to a manufacturing facility where the cell separation takes place. The antigens and other raw materials are being provided through that cell culture to create the antigen loaded dendritic cells which are then delivered back to the clinic or reinfused back into the patient.

Now the process that Dendreon's been working on really had its biologic validation, if you will, by studies that were done at Stanford by

Frank Shale and Ron Liedeslat, together with Ed Engelman looking at dendritic cells that had been isolated from peripheral blood, pulsed with idiotypes specific to the B cell lymphoma that they were studying in those patients and the results were quite striking. They clearly were able to induce very profound cell mediated immunity against idiotype and they found that cell mediated immunity associated with clear clinical benefit in a number of these patients that had failed all other forms of therapy.

The process is one whereby the dendritic cell is isolated or precursors are enriched by two buoyant density steps, the first one which is primarily to remove blood cells and granular sites and the second to deplete the culture primarily of monocytes so you have a high density fraction of cells which is then carried forward into a culture in the present of antigen over 40 hour period, harvested and then antigen pulsed dendritic cells resulting from that.

Some of the modifications that we've done to that original concept that was described at Stanford was to really look at a means by which we could attempt to close this process and actually

make it as controlled as we possibly could so we have a buoyant density medium that we work with which we've used in a variety of therapeutic settings. It's a serum-free process. There's no exogenous cytokines that we add. It's readily scaled up for the treatment of patients and it's using sterile disposable medical grade devices throughout, again, with an eye towards creating a process that you could imagine once effective in the clinic, being used commercially.

The principle of density separation is familiar to everyone in the audience, I'm sure, but it's a means by which a collection of cells can be overlayed onto a buoyant density medium following centrifugation, light cells float, heavy cells sink and you can separate the light cells from the heavy cells in this particular device by simple decantation because of this insert which creates an airlock between the two chambers.

The process in action is one also that I wanted to note is one that we can minimize the open manipulation of cells by the kind of connections between the blood and the buoyant density solution in the container and the size of this container with tubing and the size of this container is such that

we can actually process up to 50 billion cells at a time or the cells that would be contained in a full apheresis unit.

This is demonstrating the loading of the cell preparation and to the upper chamber of the device. This slide is showing the underlaying then of the buoyant density material creating a sharp interface between the solutions. This is showing following centrifugation. The red cells are now located in the lower compartment and the interface cells are in the upper compartment and then showing simple removal of that particular fraction of cells by decantation in the setting. So the time line that the leukophoresis product as delivered to cell processing facility over the first three to four hours. The two buoyant density steps were performed, resulting in the establishment of the cell culture which is then -- goes in the incubator for a period of 40 hours at which time the cells are harvested and washed and returned to the clinic for reinfusion into the patient.

The process of leukophoresis is performed in an approved facility in accordance with SOPs that we monitor as well as qualify. The processing facility that we're working in is Class

10,000 clean room and all open steps are being performed in Class 100 biological safety cabinets. The separation again uses disposable medical grade devices to maintain stability and then we have a full panel of final product QA and QC release tests that I'll get into in just a minute.

Clearly, the process of incubating these precursors for 40 hours results in the differentiation of dendritic cells into mature cells that have the hallmarks of the dendritic cells that we've heard a lot about today already. The process by which this occurs is dependent on the composition and concentration of the culture inoculum as well as the nature of the culture surface and material and a lot of studies have been done to define those before we took these products into the clinic. But clearly one thing that we do see is if you look at the culture prior to its incubation period and then look at it 40 hours later, you see cells that have greatly increased their display of CD40 and CD54 and a huge increase in display of HLA-DR. Unless you can quantify and actually characterize the cultures after the 40 hour incubation as having a population of cells that clearly display many of the markers that we've already heard about today that are

associated with the capacity of these cells to potently interact with naive T-cells.

If you purify those cells from culture you can also establish in vitro that purified dendritic cells together with naive T-cells and antigen will result over time in cultures that express high levels, excuse me, I'm missing a slide, but the dendritic cells will show over time that they've greatly increased in their capacity to stimulate a mixed lymphocyte reaction. Again, MLRs are used frequently as a measure of antigen-presenting cells in vitro and what's shown here is the slide I just started to describe, but clearly if you put up dendritic cells together with naive T-cells and antigen in culture, you see very high levels of IL12 production and gamma interferon production in these cultures which is a hallmark of the TH1-type response.

And if you look then at some of the lymphoid preparations that we've made from patients that have undergone infusions of antigen-pulsed dendritic cells we can see the same phenomenon that in the lymphoproliferative assays that we do in these patients that we see the cytokines produced are gamma interferon. There's no L4 that we can

detect, again reflecting a Th-1 type response that we've engendered in those patients.

The characteristics then are the cells we've manufactured that there's a functional maturation. There's an increase in MLR activity. They're capable of stimulating naive T lymphocyte responses. In most of the studies I've just showed you, they were KLH, but we looked at a variety of both peptide and protein antigens in vitro and the type of response that we can readily measure is a Th-1 type response, the CF regulation of cell memory molecules that are associated with potent antigen presenting cells and morphology, although I don't have a morphology slide, is consistent with the kinds of cells that we've seen in the previous speakers.

Now if we look at the question of potency testing, clearly assays like the mixed lymphocyte reaction is an assay that's a seven day assay. The manufacturing process that I've just described is one whereby we're harvesting the cells in the morning of the third day and we're delivering those cells back into the patient within six hours after that time. So the assay that requires a seven day incubation or an assay that might be a more

antigen-specific assay is really an assay that is not amenable as a release task. We've actually been doing MLRs in all the studies that we have in the clinic at the moment, just so that we can collect that data, but it's not an assay that's truly that useful to us in a manufacturing scheme to actually release the product by.

Now what I'd like to suggest in the next couple of slides is an alternative way of actually looking at some of the co-stimulatory markers and their expression and picked CD-54 by means of example, that it not only serves as a means by which we can quantify dendritic cells that have been induced to maturate in these cultures, but it's also a marker that is associated with the potency and capacity of these cells to actually interact with naive T-cells. CD-54 in this slide is showing that in an in vitro induction of a naive T-cell response against KLH, but you can completely inhibit the capacity of these cultures to do so by inclusion of monoclonal antibodies directed against CD 54. Controlled antibodies do not do that, indicating that clearly

CD-54 is involved in naive T-cell priming which has certainly been known for some time. It's a robust

stain. It is a stain that allows us to readily quantify the number of cells in our cultures. It's reliably regulated in all of the patient samples that we've worked at. Clearly, the sorting experiments that we've done have indicated that all of the cells that we are at least responsible for the kind of activity we're seeing are in that 54 positive cell population.

If you look then at -- this is kind of a busy slide, but it shows that the process of manufacturing is one that we control throughout by the system that we've designed to minimize the exposure of our cultures to any exogenous agents and then we do in-process testing which includes monitoring cell counts and viability throughout the process. We do in-process sterility sampling periodically to insure at the time of release that the product, there's minimal likelihood that there's be any microbial contamination. We also take samples in all of our clinical testing and send out for mycoplasma testing in addition to microbial testing and endotoxin, and are currently looking at our final release testing that would include phenotype analysis by FACS, endotoxin release testing at the time of harvest. Gram staining is a

test that we're doing at the moment and question whether how much longer we'll continue collecting MRL data, but it's certainly not an assay that allows us to release the product by that test. But nevertheless, it's an overall process that's one that's allowed us to introduce very reproducible a therapy into the patients that we've treated in a very reliable fashion.

Now one thing I haven't talked about in developing a therapy is that you have to have an antigen to go along with the dendritic cells that I've just talked about. By way of example, we have an extensive program in the treatment of prostate cancer with dendritic cell. Clearly dendritic cells are isolated in the way that I've just described and we're working with the recombinant fusion protein that we call PA2024 and one of the underlying assumptions that we're working with on this particular molecule is that this is a recombinant protein. It's purified by conventional purification techniques and we're assuming that it will be viewed as a well characterized biologic, even though it's used entirely ex vivo in the pulsing of these cells. But it's -- this particular antigen is related to Prostatic Acid Phosphatase. It's a prostate

specific antigen and expressed on most prostate adenoid carcinomas and we showed in our pre-clinical package to the FDA for these studies that it was both a CD-4 and a CTL target on cells expressing this antigen, but clearly, this showing three lots that we've manufactured in the vacuole virus expression system, SDS polycomyogelic phoresis is just one of many tests that we performed to establish the purity of this particular recombinant antigen and the other tests would include many of the tests that we've already heard about. It would include end terminal sequencing, HPLC and immunochemical testing.

Now we've applied this type of approach to a number of different cancers. We have an extensive program in prostate cancer. It's in Phase 2 of development. We have studies underway in multiple myeloma. Our next IND will be in breast cancer and of course, we have the historical interest that we follow at Stanford in the treatment of B-cell lymphoma.

UCSF and Mayo were the sites at which we're actually performing the antigen pulsed dendritic cell studies in prostate cancer. We've actually accrued more than 65 men to these studies

at this point. We're encouraged by in all patients that we treated so far that we can readily detect the presence of the lympho proliferative response to the antigen that we're treating and we're also seeing that as we complete our studies in Phase 2, starting to talk with the FDA about what the design of appropriate next stage and larger studies would be.

We have an extensive program in myeloma which has accrued more than 24 patients at this point. Dr. McKenzie is our principal investigator of this study. He recently presented a poster of this work at the ASH meetings last weekend where he was showing in the first 13 valuable patients that have come out, we've actually seen 6 idiotype specific responses in those patients which is very encouraging and very much like what we've seen at Stanford in Ron Levey's lab.

Clearly, in the clinical summary, we find that dendritic cell therapy is very safe and nontoxic. We've treated almost 100 individuals at this point. We've given more than 250 infusions of cells and really have a very well tolerated process that we're looking at. There's no product related

adverse events which is consistent with the general safety profile for many of the vaccines.

The dendritic cells are immunologically active in vivo. We were able to measure the immune responses and we're starting to see hence that that immunological activity may actually be associated potentially with a clinical benefit though. It's too early for us to really assign any clinical benefit at this point.

In looking forward, we actually are in Phase 2 with prostate. Multiple myeloma is in Phase 2. Breast cancer we'll be submitting IND within the next four months and then we recently licensed an antigen from the Ludwig Cancer Institute called the NYISO-1 antigen and we will see, hopefully, an IND filed on this particular product within the next 12 to 16 months. So we're looking at applying the same approach to as many cancers as we can over the next couple of years.

Thank you very much for your attention.

DR. STEINMAN: Okay, we're going to move along to Glenn Rice from Cytokine Networks to discuss fibrocytes -- there you are -- and novel antigen presenting cell.

DR. RICE: I too would like to thank the organizers for the opportunity to speak today. I was a little hesitant initially. The field of fibrocytes is, as you will appreciate, much more immature than that of dendritic cells. Hopefully, I can give you a flavor of some of the potentials for fibrocytes today throughout the talk.

What I thought I'd do is first start by basically a historical perspective of how fibrocytes were originally discovered. This work is primarily the work of investigators at Picower Institute for Medical Research in Manhasset, New York, primarily led by Rick Bucola who is a Director there.

Rick Bucola and his co-workers were studying wound response in tissue remodeling in scar formation in mice using a frequently employed model of wound healing responses which involved a cylastic tubing that is inserted in the flanks of mice which allows for one to periodically go in and withdraw cells, fluid from the tube. This is a picture of the cylastic tubing and it's filled with this polyvinyl sponge, polyvinyl soaked alcohol sponge in the middle. That's a dime on the left. And what Rick and his colleagues found that while studying the acute cellular responses involved in wound

repair, they noted a large number of adherent and spindle shaped cells that rapidly migrated in with a number of other inflammatory cell types. Initially, within the first 24 hours and it represented about 10 to 20 percent of the total inflammatory exited inside the chamber.

Now unlike your typical fibroblasts, these cells as shown by flow cytometry, express both CD-34 and Collagen 1 staining on the cell surface. This is an EM of a fibrocyte taken by Rick and his colleagues. Morphologically very distinct from circulating leukocytes and display a prominent cell surface projections intermediate in size between microvilli and pseudopodia. Cell surface phenotype has been extensively characterized by a number of investigators, refocus that a little bit, using flow cytometry and as you can see this is a positive expression for fibrocytes. They express both Collagen 1 and Collagen 3 expression on the cell surface. They have an expression on the leukocyte common antigen, CD-45 RO. They also express CD-34 as I mentioned and they surprisingly had a number of molecules that are associated with antigen expression including MHC Class 2, CD-11A, -54 and -58.

Now just as important, negative phenotyping. They did not express a number of markers for monocytes and macrophages including esterase, CD-4, CD-14 and CD-16. They do not express T-cell receptor. They don't express markers for epithelial cells. A Von Gullabran factor, endothelial cells is negative. Alpha actin for smooth muscle cells is also similarly negative. Also CD-83, not shown here, is also negative.

Now to assess the capacity of fibrocytes to present an antigen, autologous T-cell proliferation assays were performed and T-cells were purified from peripheral blood from tetanus toxoid immunized individuals and stimulated in vitro with tetanus toxin together with fibrocytes shown in the bottom panel as APCs.

You can see with relatively low number there's a vigorous response to the soluble tetanus toxin in the cultures. Now with the same T-cell preps, dendritic cells and monocytes were compared as has been found by a number of investigators. Monocytes give very poor response. Dendritic cells may give slightly higher response than fibrocytes, but they do so at higher cell numbers.

Now the functional requirements for

HLADR-86 and -11A and -54 were determined by adding antibodies prior to the stimulation and you can see that each of these antibodies was sufficient to block most of the response.

Now the functional responses of fibrocytes isolated from mice were also compared. Fibrocytesare fairly difficult to isolate from mice as I'll talk about a little bit more in detail later. But in this case, mice were immunized with who AIDS, HIV antigens, proteins GP-120 or p24. Five days later, the fibrocytes were isolated and pulsed in vitro with the corresponding antigens and you see a nice vigorous response to in vivo immunization.

Now priming of naive T-cells has been considered a function of professional APCs and to test the ability of fibrocytes to prime naive T-cells in vivo, fibrocytes were pulsed in vitro with either p24 or gp120, washed, injected into the foot pads. Five days later, proximal lymph nodes were isolated, associated and pulsed with either gp120 or p24.

As you can see with the p24 response, pulse in vitro, there's a very vigorous response of p24 and this can be blocked by adding immuno

magnetic selection of CD-4 cells prior to stimulation. TP-120 pulsed cells, gp120 immunized animals with p24 response showed no response. And similarly gp120 showed a vigorous response.

Now that antigen pulsed fibrocyte, we're not simply transferring antigen to other host APC types was established by experiments which used antigen pulsed fibrocytes from two parental strains which were injected into F1 offspring mice. T-cell reactivity of F1 offspring is confined predominantly to antigen presented by one of the parent strains and priming and re-stimulation to APCs must share the same haplotype, so in this experiment, F1, that is d times k, mice were injected with pulsed fibrocytes from either parent, d or k, and five days later the popliteal lymph nodes were isolated and depleted of endogenous Class 2 MHC by immunomagnetic selection. The F1 APC depleted lymph node cells were then cultured with F1 cells were parent strained d or k and swing cells as a source of the APC, plus or minus gp120.

So the F1 APC depleted lymph node cells were reactive to antigen in the presence of F1 re-stimulation APCs when priming with fibrocytes from either parent. However, if a parental strain

was used as a source of re-stimulation, APC, the F1 APC depleted lymph nodes would only proliferate if the priming fibrocytes were from the same strain. So fibrocyte priming and APC re-stimulation only occurs in the setting of a shared MHC haplotype. Thus, fibrocytes do not merely function to deliver antigen to other APCs, but rather act to directly sensitize naive T-cells in an MHC dependent manner.

In addition, if one stains fibrocytes with a red linker dye and injects them into the rear footpads of mice and 24 hours later examines the popliteal lymph nodes, they're actually about 5 percent of the injected fibrocytes can be found in the lymph nodes as shown here.

Now the cytokine and growth factor profile of phenotype expression of fibrocytes is interesting and this is a way to estimate that using a PCR so these are either immuno magnetic selected T-cells, monocytes or fibrocytes and we're comparing in this case the control CD-3. Of course, only the T-cells will express CD-3, CD-14, of course, only on monocytes; CD-34, fibrocytes. Collagen 1, as I mentioned earlier, is a marker we use quite extensively for fibrocytes as shown here, expressed only in the fibrocytes following this column here.

TNF, both in the monocytes and the fibrocytes, IL-1 beta in both. Interestingly, IL-10 is found in the fibrocytes, at least at the MR and A level.

Fibrocytes express a number of different chemokines, including MIP-1 alpha and beta, both regulatable by IL-1, MIP-2 and the interferon inducible chemokine as well.

PDGF alpha, as well as FGF, not shown here is also expressed by fibrocytes, TGF beta, MCS, but not gamma interferon. Many of these are regulatable by growth factors that one commonly finds in wound healing settings.

Just one final historical kind of slide. Additional evidence of the role of fibrocytes in antigen presentation in vivo was obtained by immunohistochemical staining of human cutaneous scar specimens. Now these cutaneous, human cutaneous scar specimens were examined in the presence of -- we examined for the co-expression of either CD-34 and HLADR and DR was expressed with alkaline phosphatase and is shown as red and CD-34 is brown and granular. But you can see these co-expression not as clearly here as under the microscope, but there's clearly a number of cells, spindle shape

morphology that express both markers in cutaneous scar tissue.

This is a higher magnification of one such cell showing, in fact, cytoplasmic projections of

CD-34.

So one other -- throughout the talk I'll kind of try and differentiate fibrocytes from dendritic cells. I think the phenotype is one aspect. The flow cytometric phenotype is one aspect. The morphological phenotype is another. These are attached cells. They're spindle shaped cells typically, but also interestingly they also will proliferate in culture. This is work done at the Picower Institute showing that they have about a 48 hour, in this case, doubling time.

Our clinical studies have shown that the proliferation is probably for the most part, the most donors, somewhat more extended than 48 hour doubling time, but they do appear to proliferate. In fact, you very frequently can find in cultures at later stage cultures, 18, 21 and 25, these proliferating foci or parent proliferating foci cells of adherent cells in the cultures.

This is just another shot of cells generated from patients showing the long spindle formation.

Okay, so in terms of clinical applications, cytokine networks was interested in at least initiating a pilot Phase 1. The idea here is to look for these isolating cultures of fibrocytes which I'll describe our process in a moment, and our process for antigen presentation and of course, re-vaccinate. The issue of antigen presentation, I put this in as kind of a reminder to also differentiate fibrocytes from dendritic cells. This is infection of, on the top, dendritic cells or fibrocytes with either a macrotrophic, but also, not shown here,

T-trophic HIV virus. This is work done at the Picower Institute showing infection of dendritic cells and lack of infection with fibrocytes. These data and others have led to an AIDS initiation grant by Nancy Haywood who is the PI at Seattle Biomedical Research Institute, using fibrocytes as a means to generating immune response against HIV in macaques at the present time.

We were interested in oncology and as has been mentioned earlier, there are a number of

ways to prime APCs for specific antigens, one of which is peptide pulsing. This is FITC-dextran uptake of various sizes, I'm sorry you can't see it very well, 3,000, 4,000 and 500,000 molecular weight. The right panel is fibrocytes. The left panel is the control cell THP-1. And you can see relatively quickly within about an hour, there's significant uptake of these FITC-dextran labeled cell beads by the fibrocytes. So they're highly phagocytic and potentially could be utilized in a peptide pulsing type of antigen loading.

This is work by Drs. Jhong and Kufe at Dana-Farber, who used a biorad, ballistic gene gun using DF-3 MUC-1 CDNA on fibrocytes with psv-MUC-1 expression. The top is immunohistic chemical staining of MUC-1 expressing cells in a mock transfection, fibrocytes in a mock transfection and those shown lower are those expressing MUC-1 48 hours after gene gun delivery. You can see a very surprising and potent high level expression, both in terms of amount of protein expressed per cell as well as the overall percent of cells expressing MUC-1. So I think this could also be an interesting application for antigen loading of fibrocytes and this is also, I think, for those of you who have

tried transfection methods in dendritic cells further elaborates differences between fibrocytes and dendritic cells.

We chose as the initial pilot Phase 1 to work with Jim Mulé's technology of tumor cell lysate pulsing. This is work he'll describe in detail tomorrow, but just some of the salient advantages of such an approach. It has a greater potential for augmenting broad T-cell responses to tumor associated antigens, plus potential for escape. Greater potentials that trigger T-cell reactivity to rejection antigens. There's evidence that both helper and

CTL- defined epitopes have increased presentation. You can circumvent the need for fresh viable tumor cells in this type of approach, and of course, you need for molecular characterization of the antigen.

As I mentioned earlier, it's extremely difficult to isolate fibrocytes from mice. Multiple cardiac punctures are required in many, many animals to get reasonable numbers to use for vaccination, but this is one such experiment in tumor model of MCA-207, fibrosarcoma, a nonimmunogenic model. We have similar data. We've done this multiple times and also with D5 B16 melanoma, similar data. This

is a challenge experiment where the animals are pulsed, are vaccinated three times on weekly cycles prior to challenge with tumor and you can see that those animals are pulsed with, are vaccinated with lysate-pulsed tumor, show a decreased response rate. The reason this is coming down is actually the tumors are fairly necrotic with time, get increasingly necrotic with time. So the Phase 1 clinical trial at the University of Michigan that is on-going now with Dr. Chang and Dr. Mulé is the dose escalation up to 10^8 cells every two weeks for up to three vaccinations. The cell processing involves apheresis and the cells are pulsed for 24 hours with autologous tumor lysate and we co-administer KLH as a surrogate. Patient inclusion, as shown here, a variety of advance stage tumors. Primary end point is toxicity, but of course, we really have our eye on the secondary endpoints which a variety of immune responses including DTH, both to KLH and autologous tumor,

T-cell subsets, both in peripheral blood and lymph nodes and a variety of T-cell assays.

The method of isolation is very fairly straight forward. It involves isolation of the whole blood Ficoll-Hypaque plate onto fibronectin

coated plates, washed with media 24 hours later and basically go for 21 days on these fibronectin coated plates. We've optimized the media. It's OPTI-MEM. We're still using -- now we're using 10 percent human AB serum. We have not converted this to serum-free media yet.

In addition, well then at 21 days, the cells are pulsed for 24 hours with irradiated tumor lysate and then injected into the patients. The yields are typically 2 to 7 percent, i.e., in cancer patients. And I also mention that there are no cytokines of growth factors required in this process as shown now.

Just two slides showing just preliminary data. This is allogeneic. In antigen-specific tetanus toxin MOR activity in fibrocytes isolated from one cancer patient, you can see there's reasonable activity using APCs as -- or MLRs, a surrogate assay. Now unlike the previous talk, we had to have 21 days, so the cells are actually processed in University of Michigan and sent to a facility in San Francisco where we perform MLRs, one such surrogate assay. FACS analysis is another. This includes positive Class 2 86 and Collagen 1 staining with CD-14 exclusion. This is just a panel

of allogeneic MLR for the cancer patients. You can see reasonable stimulation for the patients as a surrogate. And of course, we check for KLH responses in vitro and even after two re-stimulations there is significant activity to KLH by the fibrocytes, so they can certainly display KLH and present KLH.

This is a 10,000 square foot class -- well, cell processing facility in south San Francisco. It has a variety of cluster units, including three cluster at Class 10,000 clean rooms and four other clean rooms that are also Class 10,000.

Since the fibrocytes at present are an open system, we culture single patients in each of, right now about 35 incubators.

So in conclusion, I just hope I've given you kind of an overview, historically and scientifically, of the fibrocyte, both mouse and human fibrocytes express surface components required for antigen presentation. They induce strong allogeneic and antigen specific T-cell proliferation. Some applications that may be useful clinically, they're easily transfected. They rapidly take up and phagocytose peptides and

proteins. They can prime naive T-cells and they're sufficient to inhibit tumor challenge to a variety of models and this has led to initiation of a Phase 1 clinical trial pilot at University of Michigan.

I'd like to just acknowledge two key collaborators at Picower Institute, Rick Bucola and Jason Chesney, Jim Eulay and Bruce Redman at University of Michigan and Dr. Clersa Nasker who is part of the audience today. I'd like to especially note her contributions to this clinical trial.

Thank you.

(Applause.)

DR. STEINMAN: Thanks, Glenn. Our next speaker is scheduled to be Malcolm Mitchell, but none of us have seen Malcolm. Is Dr. Mitchell here? Okay, in that case we're going to take an early break and start back again at 2:45. Please, no later.

(Off the record.)

DR. MULÉ: It's a pleasure to introduce Dr. Donald Kufe from the Dana-Farber and he will be speaking today on dendritic cell tumor cell fusions and presenting some recent data both preclinically and more importantly in the realm of human tumors in preparation for a clinical trial.

Dr. Kufe?

DR. KUFE: Good afternoon. Dendritic cells pulsed with peptides and proteins derived from tumor antigens, as we've heard today, induce potent specific anti-tumor immunity. In the absence of an identifiable tumor antigen which is the case in most human tumors, dendritic cells have been pulsed with tumor cell membranes, LU-8s from tumor cells and as we heard about this morning, apoptotic bodies.

We've explored a different approach using dendritic cell-based vaccines by fusing dendritic cells with tumor cells and thereby forming heterokaryons that express tumor-associated antigens as well as the machinery needed for the activation of T-cells.

Using this strategy, expression of tumor-associated antigens as well as yet unidentified tumor antigens are processed endogenously and expressed by MHC Class 1 in the context of
co-stimulatory molecules.

We developed this approach to induce active specific immunotherapy against an antigen that we identified, we and others identified in the early 1980s. This antigen is known as MUC-1 or

mucine 1. It's a human carcinoma-associated antigen and as background this antigen is over expressed about 30 to 50 fold compared to normal secretory epithelium in the majority of breast carcinomas, as well as carcinomas derived from diverse other tissues.

Moreover, in carcinomas, MUC-1 exhibits an altered cell distribution and aberrant glycosylation patterns. For example, in this section of an infiltrating ductile carcinoma stained with immunoperoxidase, MUC-1 antigen expression is at high levels throughout the transformed epithelium compared to the minimal expression seen here along the apical borders of the cells lining the ducts and in these cells MUC-1 antigen is expressed only along that portion of the cell that lines the duct whereas in the transformed cells, MUC-1 antigen is expressed throughout the cytoplasm and on the entire cell surface.

As a result of cloning the MUC-1 gene by my group and by others in the late 1980s, the structure of the antigen is shown schematically here. There's a cytoplasmic tail involved in intracellular signalling, a transmembrane domain and an extra cellular domain that consists of highly

conserved 20 amino acid tandem repeats that range in number from 50 up to 100. These tandem repeats are heavily glycosylated, such that the antigen extends beyond the glycocalyx as a rigid rod-like structure.

Now interest in MUC-1 as a target for active specific immunotherapy is obtained in part from the over expression of this antigen in diverse carcinomas, but also the finding that MUC-1 is recognized by antibody and CTL responses in patients with breast cancer as well as other types of carcinomas and even in healthy multiparous donors.

Now in cancer patients, the finding that MUC-1 is recognized by CTLs indicates that the antigen can be recognized by the immune system, but the response is insufficient to clinically alter the course of the disease. Consequently, the hypothesis has been that one could increase the response, immune response against MUC-1 and thereby induce clinically active immunity.

So to induce active specific immunotherapy against MUC-1, we developed an approach involving the fusion of MUC-1 positive tumor cells with dendritic cells such that the heterokaryon that forms expresses MUC-1 as well as other yet unidentified tumor antigens in the context

of Class 1 as well as Class 2. The heterokaryons also express co-stimulatory molecules and adhesion molecules necessary for the activation of naive T-cells and thereby the induction of cytolytic T-cells.

The efficiency of the fusion cell procedure can be demonstrated using bi-directional flow cytometry, in this case using a FITC-labelled antibody against MUC-1 and a PE-labelled antibody against MHC-2. The dendritic cells express Class 2, but no detectable MUC-1 antigen. The carcinoma cells in this mouse model stably expressing the MUC-1 gene, expressed MUC-1 antigen but no Class 2. If we add -- mix the dendritic cells with the MC-38 MUC-1 carcinoma cells, and incubate for six days, the cell populations remain distinct. That is, one population, the dendritic cells expressing MHC Class 2 and the tumor cells expressing MUC-1.

However, if we briefly expose these cells to polyethylene glycol and induce a fusion, after two days of incubation the fusion cells express a small percentage of cells that have both Class 2 as well as MUC-1. By six days over 20 percent of the cells expressed both antigens and by

10 days, 50 percent or more of the cells expressed both Class 2 and MUC-1.

The morphology of these cells, that is the dendritic cell -- sorry, the dendritic cells shown here, the carcinoma cells shown here and the fusion cells are shown here. The fusion cells retain some of the characteristics of the dendritic cells with dendrite-like extensions. The fusion cells also express other antigens common to the dendritic cell. For example, in addition to Class 2 as seen on the fusion cells, not expressed on the tumor cells, the fusion cells also express B7-1, B7-2 and ICAM-1. So these cells then express both the tumor associated antigen, as well as molecules necessary for presentation of antigen, co-stimulation and adhesion.

Now Jianlin Gong in our laboratory showed last year that one could immunize mice with fusion cells and induce anti-MUC-1 immunity as evidenced by the production of antibodies, as well as CTLs that were specific for MUC-1. Moreover, immunization with the fusion cells prevented the growth of tumors in challenge experiments, but more importantly, these fusion cells were effective in

eliminating established pulmonary metastases of the MC-38 MUC-1 carcinoma cells.

Now in more recent studies, we've turned to a transgenic mouse model which expresses the MUC-1 antigen. It expresses human MUC-1 in a pattern, tissue pattern that's identical to that scene in man. Moreover, as I showed you earlier in the human breast epithelium, in the transgenic mouse model, MUC-1 is expressed along the apical borders of the cells lining ducts, in this case, bronchioles and in this case mammary ductules. There's a fine rim of staining along the apical borders of these cells, staining specific for human MUC-1 antigen as confirmed by peptide blocking.

So this animal model, this transgenic mouse model provides an opportunity to assess the immunity induced by fusion cells or other approaches and indeed the MUC-1 transgenic mice are immunologically unresponsive to challenge with purified MUC-1 antigen mixed with adjuvant or to challenge with irradiated MUC-1 positive tumor cells. So we use this model to determine whether the fusion cells could reverse immunologic unresponsiveness to MUC-1 in these animals.

Now we use a tumor model where MC-38 carcinoma cells expressing MUC-1 are injected in the tail vein of these MUC-1 transgenic mice. Multiple pulmonary metastases are established and grow progressively and ultimately kill these animals. If, however, we establish these preliminary metastases and within seven days treat with the fusion cells, all of the animals as shown here of 10 lungs from animals with previously established pulmonary lesions, all of these animals are rendered disease-free and these animals are long-term survivors.

Importantly, this irradiation of MUC-1 positive tumor cells is associated with an antibody response that's specific to MUC-1 and a CTL response. Now as I pointed out earlier, these animals express MUC-1 on normal tissues, on the apical borders of secretory epithelial and a key question was whether the induction of anti-MUC-1 immunity using these fusion cells contributed to autoimmunity. Indeed,the animals lived a normal lifespan without evidence of disease and analysis of MUC-1 positive tissues, for example, here in the bronchi where we see MUC-1 staining along the apical borders lining the bronchi, we see in the animals

that were rendered disease-free with the fusion cells a similar pattern of expression, so the antigen remains expressed. There's no destruction of the epithelium. Moreover, there was no evidence for infiltration of these tissues with CTLs.

So we can summarize our findings with this slide, using the fusion cell vaccine. We induce

anti-MUC-1 immunity as evidenced by specific CTL and anti-body responses. We induce anti-tumor activity that's directed against MUC-1 and there's no evidence for autoimmunity.

Now I should point out that this induction of anti-MUC-1 immunity is only in part responsible for the irradiation of the tumor cells in that one can fuse MUC-1 negative cancer cells MC-38 cells with dendritic cells and use that as a vaccine and eliminate MC-38 MUC-1 negative pulmonary metastases. So as I pointed out earlier, the fusion cell vaccine offers the advantage of inducing immunity against known tumor antigens, in this case, MUC-1, as well as yet unidentified tumor antigens.

Well, since the original paper published by Jianlin Gong in 1997 using the MC-38 carcinoma model, several other reports have appeared using the

fusion cell approach, one with a mastocytoma, another paper using B-16 melanoma and Lewis lung carcinoma and then the third paper using B-16 and a lymphoma that's defective in the presentation of peptide antigens.

Now one point should be made. First of all, in each of these studies the fusion cell vaccine was effective in eliminating established tumors. An additional point, in this particular paper, the incubation of B-16 melanoma cells with dendritic cells, without actually fusing the cells with polyethylene glycol was sufficient to confer upon the dendritic cells the induction of antigen specific immunity.

As I showed you earlier, in our studies where we mix dendritic cells and MC-38 carcinoma cells, we were not able to achieve a fusion of the cell membrane without a first incubation with polyethylene glycol. So it may be possible to just co-incubate and as we saw earlier today, co-incubation where uptake of apoptoticbodies is in part sufficient to induce immunity. In certain settings, particularly MC-38 and some of these other tumor models, one has to take it further by actually fusing the cell membranes together.

Well, these findings have suggested that the fusion cell approach is applicable in diverse types of tumors, carcinomas, melanomas and lymphomas and to determine then whether this approach is potentially applicable to human dendritic cells and human tumors, we pursue fusion using human breast cancer cells. In fact, most of our efforts thus far have been focused on breast cancer cell fusion from the breast cancer cells that have been derived from primary as well as metastatic lesions, they're shown here. These are the human peripheral blood dendritic cells that you've heard about as cultured in GMCSF and IL-4. And here are the fusion cells. Here, we're standing with an antibody against MUC-1. The tumor cells exhibit that pattern of high levels of MUC-1 expression throughout the cytoplasm and on the cell membrane and likewise the fusion cells also express MUC-1 throughout the cytoplasm on the cell membrane. The dendritic cells are MUC-1 negative.

Now early in the fusion process, shortly after exposure of the dendritic cells and this is an autologous system now where we're using a patient's own dendritic cells and her own tumor cells, you find these clusters that consist of MUC-1 positive cells and the MUC-1 negative dendritic cells and

then these cells progress to further fusions where we see here a fused cell that now is stained from MUC-1 which is the reddish color you see here, as well as Class 2, the bluish stain showing that now the tumor cells which were originally Class 2 negative, MUC-1 positive, and the dendritic cells which were MUC-1 negative and Class 2 positive, now the fusion cell expresses both of these antigens.

Now one of the questions, of course, is whether these autologous fusion cells, human DCs and human breast cancer cells fused together are functional, so in allogeneic MOR assays, we incubated dendritic cells, tumor cells and the fusion cells with allogeneic T-cells and as shown here the stimulation with dendritic cells, but not with the tumor cells and the fusion cells are also capable of stimulating the MLR reaction.

This is another preparation, again autologous dendritic cells with autologous breast cancer cells. The dendritic cells, the tumor cells, here are the fusion cells shown with the close squares stimulating T-cell proliferation. Moreover, one can irradiate these cells with lethal doses of ionizing radiation and the infusion cells remain active in stimulating MLR.

In other studies we've asked whether the fusion cells were capable of stimulating autologous T-cells and we performed this type of experiment where autologous T-cells are incubated with the patient's tumor or with autologous fusion cells derived from that patient. There's little evidence of clustering between the autologous T-cells and the tumor cells, but with the fusion cells we see these clear clusters of autologous T-cells surrounding the clumps of fusion cells and the autologous T-cells begin to proliferate. We've isolated the T-cells incubated with tumor cells and the T-cells incubated with fusion cells by nylon wool and then we've asked whether these T-cells can kill autologous tumor and as shown here in three separate instances of autologous fusion cells stimulating T-cells, the three different patients, the T-cells incubated with the fusion cells kill autologous tumor cell targets, but T-cells incubated with the tumor cells do not kill the autologous targets. This is a chromium release assay.

Moreover, we've used monocytes derived from the same patients as controls and the stimulated T-cells do not kill the autologous monocytes. Now we have not elucidated the nature of

this killing, what the antigens are, whether it's directed against MUC-1 or yet other antigens, whether it's MHC restricted or unrestricted and those studies are underway, but the preliminary evidence suggest that we can stimulate autologous T-cells using the fusion cells.

So in terms of characterizing the dendritic cells and the breast cancer cells and as well the fusion cells, in terms of identity testing, we're assaying our dendritic cells for CD-83, Class 2, B-71, B-72. We also analyze our breast cancer cells for these antigens as well as MUC-1 and cytokeratin. We characterize the fusion cells by doing bi-directional flow cytometry for CD-83, Class 2, co-stimulatory molecules, MUC-1 and when MUC-1 is negative, cytokeratin. And as I've shown you we've been testing these fusion cells and functional assay allogeneic MLR as well as CTL activities.

So we believe that we're in a position now to consider applying this to the clinical setting where we harvest the patient's on dendritic cells and fuse those dendritic cells to autologous tumor cells as a potential vaccine.

So to summarize then, we have made dendritic tumor cell fusions. We've tested this in

mouse models and have demonstrated that we can induce anti-MUC-1 immunity and we can induce immunity against unidentified tumor antigens and this has been confirmed by several other groups now in other tumor models and we have just begun to pursue this approach at least technically in breast cancer, using an autologous system.

I'd like to identify and recognize the credit for all of this work on the fusion cell vaccines, Jianlin Gong and David Avigan have been working collaboratively in the animal models and on the development on the human vaccine as well. I'll stop here.

Thank you.

(Applause.)

DR. MULÉ: Thanks, Don. It's a great pleasure for me to introduce Dr. Mike Lotze from the University of Pittsburgh Cancer Institute. Mike, as many of you know, has been a pioneer and driving force behind many of the immunotherapy trials in patients and Mike today will give us an overview of the use of dendritic cells in the treatment of cancer.

DR. LOTZE: Well, good afternoon. When I saw the title for my presentation in the schedule

that was sent out, it was clear that the organizers wanted me to focus on issues related to flt3L and so I'm going to spend a good portion of my time talking about the use of what we call FL in the murine tumor models as well as in human clinical trials. Then perhaps end with some aspects of feeding apoptotic bodies and apoptotic cells to DC, an area of current interest in many labs.

So I'd like to begin with just a brief review of the flt3 receptor and the flt3L. Flt3 was originally identified by a number of different groups as a tirasing, kinase signalling type receptor that came out of a fetal liver. It represents one of a series of different receptor tirasing kinases. It, along with KIT and the PDGF receptor represent members of the receptor tirasing kinase Class 3. Other extended members of this family include the insulin receptor, the FGF receptor, FLT2 receptor and the HER-2-NEW (sic.) receptor.

There was a big search since it was found that the flt3 molecules expressed apparently uniquely on hematopoietic progenitors for the ligand. The ligand was identified by two groups in the period between 1993 and 1995. Flt3L was

subsequently demonstrated to have profound hematopoietic effects as a legitimate stem cell factor, and furthermore, it appeared that it profoundly synergized with a variety of other hematopoietic factors to enhance survival, proliferation, differentiation and self-renewal.

And so subsequent studies done, as I'll review in a minute primarily by Eugene Maraskovsy at Immunex demonstrated that it also had a profound effect on the ability to expand cells of both lymphoid and myeloid DC phenotype. And so this allows me the opportunity to just say as an aside that there are two predominant approaches to using DCs as therapies. One, and the one which has consumed a number of individuals in this audience, including our own group is to try and find what is the best way to get tumor antigen into dendritic cells using a variety of different protocols, including the ones we just heard about from Don Keefe. But in addition, an alternative strategy is to try and find ways to enhance DC delivery directly into tumors. And there seem to be a number of ways that one might be able to do that. This would include either administration of GMCSF, pegolated

GMCSF, the administration of flt3L or perhaps even the direct injection of DCs into tumor.

And so Eugene a number of years ago demonstrated at Immunex that flt3 administration induces the accumulation of DCs in a variety of different organs, in the spleen, liver, bone marrow, etcetera. This is widespread. As many as 25 percent of the cells in the spleen have a DC phenotype. This is the appearance of a normal mouse spleen. This is the appearance of two spleens from animals treated with flt3L, a mouse treated with IL-12 and an animal with both cytokines.

The appearance of the spleen is remarkable. In normal animals, the apparent white pulp now being expanded by these large numbers of pale cells which represent phenotypically dendritic cells after 10 days of administration of exogenous flt3L.

One can also take advantage of this to use ways of separating DCs directly from either the peripheral blood or the spleen. This is just an example of this approach using DCs labeled with microbeads and then separated using magnets essentially to separate out DCs which bear individual phenotypic markers. We have done this as

a strategy to try and isolate the DC-8 alpha positive lymphoid dendritic cell from nude animals that failed to have CD8 positive T-cells as a way of enriching cells of lymphoid DC phenotype. And one can demonstrate that one can, using these strategies, generate a very pure population of lymphoid dendritic cells from the spleens of FL treated animals.

In addition, as has been previously shown with cells isolated from the thymus of animals in Ken Shortman's experiments, one can demonstrate that these lymphoid dendritic cells separated from the spleens of flt3L treated nude mice are also not very good stimulators in the next lymphocyte response, demonstrating again their different biologic activities from their nominal myeloid dendritic cell brethren.

We've done extensive studies evaluating the role of FL and murine models. I'll just briefly talk about one using FL by itself and in combination with IL-12 where animals are injected sequentially for 10 days with one or both cytokines and then a variety of studies done on peripheral lymphoid organs and then also phenotypic and functional studies done to evaluate the role of FL. As is well

known to, again many people in this audience, Fl administration allows the generation of an extraordinary number of dendritic cells. Here you can see there's about a 200 to 300 fold increase in the number of DCs that are available from the spleen compared to the number of DCs you get from a flt3L-treated animal to a normal animal, similar increases, although not as profound occur in DCs derived from the bone marrow, again a flt3L-treated animal versus an animal treated with HCC.

One can also show that these cells obtained from either the bone marrow or the spleen are functional. Here you can see that there's about a 30 to 40 fold increase in the ability to stimulate when cells are derived from the spleen of a flt3L-treated animal compared to a HCC-treated animal.

And in addition to their ability to enhance cells within the spleen, it also appears that flt3L will drive cells in a variety of different tissues. As I mentioned before, I'll just show you one in the skin studies done by Clements Esche and Michael Shurin in our group, demonstrating that if you treat animals in this instance wild type animals versus IL-12 knock-out animals, that one can

demonstrate a limited number of dendritic cells presumably Langerhans cells in the dermis, I'm sorry, in the epidermis of the mouse skin. If you treat with flt3L, you can see a marked accumulation primarily of the so-called dermal dendritic cells, primarily within the dermis of animals with less increase in the epidermis. There appears to be somewhat increased number of cells in the epidermis relative to the dermis when you treat with IL-12 and a modest additive effect in some animals when treated with both. This is actually summarized here in wild type mice comparing it to IL-12 knock-out mice and one can demonstrate again an increase in the number of dermal and epidermal dendritic cells as indicated by CD-86 positive cells with treatment with flt3L. If you treat with a combination one can demonstrate increases in both the epidermis and dermis and interestingly in the absence of IL-12 or even with exogenous IL-12, it appears that this ability of FL to drive DC accumulation appears to be abrogated, suggesting that IL-12 is necessary for this FL effect.

Now what about the use of FL as an anti-tumor reagent? This was again originally identified in functional assays in murine tumor

models by David Lynch at Immunex in a paper published in Nature Medicine in June of 1997 showing that animals treated with flt3L between Day 7 and 10 after tumor inoculation could reject established tumors. This was subsequently confirmed in a breast cancer model as well in our own group by Clements in a melanoma lymphoma model published in Cancer Research and a liver metastasis model demonstrating the important role of NK cells which just appeared in the current issue of Journal of Immunology.

This is the appearance of a spleen again, this time stained with NLDC 145 in animals treated with flt3L compared to an untreated animal showing again the massive expansion of dendritic cells in the spleen. One can also demonstrate this within a variety of different tumors. This is the MC-38 carcinoma showing again after flt3L, a massive expansion of cells within this tumor, as well as the C-3 sarcoma showing again relatively minimal number of DCs in control animals and following flt3L an enhanced number of DCs found within the C-3 sarcoma with FL therapy.

One of the questions that has been raised repeatedly is whether there might be alternative strategies to elicit an immune response

to tumor. This would perhaps include, rather than the ex vivo culture of tumor or of dendritic cells with GMCSF and IL-4, the in vivo administration. We've done a limited number of studies. I'll just show you one where we've directly compared the ex vivo administration of IL-12 and 53 Ligand with various combinations of GMCSF and IL-4 that were made available from Schering-Plough. Here you can see in the C-3 sarcoma these are each line is a separate animal, tumors growing progressively and if you administer IL-12 at a microgram per day beginning on Day 7 one can demonstrate a complete elimination of tumor. Similarly, one can demonstrate that with flt3L, these animals are immune to a subsequent challenge with tumor, with modest, but not absent effects, of GMCSF, IL-4 or the various combinations. You can see in this very sensitive tumor model that there does appear to be some anti-tumor effects, but the best anti-tumor effects are again those mediated by simple administration of IL-12 or flt3L.

I'd like to briefly review some of the studies done in the clinical arena. These slides were made available to me by Donna Caron and Mal Lepsik, representing the results of the flt3L Phase

1 study which is a randomized double blind study utilizing daily sub cu. injections for a maximum of 14 days with escalating doses of 5-3 Ligand as well as in some groups GCSF, given 10 micrograms per kilogram per day, as well as a placebo controlled group.

The administration of flt3L to humans was associated with very modest toxicity, only grade 1 or 2 in severity. Primarily, injection site reactions, as well as an occasionally painful and enlarged lymph nodes, that were in our experience spleen, but all of these resolved without serious sequelae and there appears to be little in the way of adverse events associated with the simple administration of FL.

Like the murine models, there appears to be enhanced numbers of dendritic cells. These include not just the CD-11 C positive, but as I'll show you later, CD-11 C negative dendritic cells presumably representing the lymphoid dendritic cell subset in the human. In addition, there appears to be an overall expansion of myeloid cells and cells that appear like these large macrophages that Jacques showed you earlier.

So if you examine administration of flt3L here over 14 days, here in three separate individuals, you can see on a logarithmic scale that there is an increase in the number of dendritic cells which peaks around Day 10 to 14 and levels off. These cells that increase as a percentage of cells in the peripheral blood, you can see upwards of a 10 to 50 fold increase in the total number of dendritic cells in the peripheral blood. This is with an N of 15 compared to a placebo controlled group. Similarly, if you look at the absolute number of dendritic cells, here you can see again, again this is on a logarithmic about a 50 fold increase in the number of dendritic cells in the peripheral blood of patients receiving exogenous flt3L.

What are these cells and what are their functional capabilities? If you sort on the CD-11C positive CD-14 negative cells one can demonstrate that these cells not only have an enhanceability to stimulate in an autologous MLR shown here in terms of background counts, but also have the capability of presenting exogenous antigen here in this instance tetanus toxoid, ovalbumin KLH and hepatitis B and that these cells again appear to be the

predominant cells capable of this activity. The CD-11C positive CD-14 positive macrophages appear to have far less activity, again demonstrating this remarkable difference between nominal dendritic cells expanded here in the instance of FL administration comparing to the macrophages.

If you examine the phenotype here in three separate individuals of these DCs, this is again showing a placebo. Looking at CD-86 in the abscissa and along the ordinate, a cocktail of antibodies reflecting a variety of different cellular subsets to try and drive them all out of the lower right hand corner. You can demonstrate a relatively small number here estimated as being 2.6 percent which is probably a high estimate, but you can show that in three separate individuals you can drive this percentage up to a considerable degree, 25 percent, 17 percent, almost 20 percent by 14 days of administration of flt3L.

If you look at another phenotypic marker in this instance using the myeloid marker CD-33 which is on both macrophages as well as dendritic cells and here again use the same cocktail of antibodies in the ordinate, one can again demonstrate in a placebo treated individual about 10

percent in this particular individual, the cells appearing to be CD-33 positive and probably CD-14 positive. You can demonstrate that there's an increase in this cell population as well, 21 percent, 25 percent or 22 percent in patients treated with flt3L, in addition to the increase in the nominal dendritic cells. And so it appears that not only are these cells increased, but also other cells of myeloid origin.

This looks at Class 2 and this has been demonstrated repeatedly at this conference. Once can enhance very high expression of Class 2 as a characteristic of immature and further increased on mature dendritic cells and one can again demonstrate a large increase over a placebo controlled individual, in a phenotype or lineage negative cells expressing Class 2, again nominally DCs as well as an increase in these other myeloid cells presumably macrophages.

Let's see, as I mentioned there's an increase not only in the CD-11C positive DCs, but if you gate on large cells one can also demonstrate that there's an increase in CD-11C negative DCs. Here, one can demonstrate in normal individuals as a percentage, a very low number of these cells. Here,

you can see with flt3L prior to treatment and after treatment, again an expansion as a percentage and as an absolute increase probably about 10 fold, less expansion than the overall DC numbers associated with FL administration.

This is the picture of one of the phenotypic markers, nominally associated with lymphoid dendritic cells, the IL-3 receptor or CD-123 which has been defined by a number of groups as being characteristic of the lymphoid DC, particularly in the human. Here you can demonstrate that there is a increase in the number of lymphoid dendritic cells again shown as an increase in the number of cells expressing the CD-123 marker.

If one examines these different cellular subsets examining CD-14 versus CD-11C and these are broken down into four different definable subpopulations, those that are CD-11C positive, that are CD-14 negative, those that appear to be double positives and those that are weakly positive for CD-11C, one can demonstrate that the ones which appear to be greatest in terms of their stimulatory activity include those that are CD-11C positive and CD-14 negative, again confirming that this is the phenotype of the most active stimulator within the

mixed lymphocyte response, very comparable to what one would see with a CD-1A positive bone marrow derived dendritic cell. And that cells derived from this population or this population are less stimulatory in a mixed lymphocyte response.

We've just completed a Phase 2 study in patients with melanoma. This represents a study conducted at three centers, University of Pittsburgh, University of Minnesota and at M.D. Anderson. We are just going over the data now. I cannot present the clinical results, but we'll say that even in melanoma patients there does appear to be an expansion of lineage negative DR positive or CD-86 positive cells. Normally, in our experience this represents .3 to 1 percent of the cells and you can see reproducible in melanoma patients treated with flt3L an increase to 6 to 10 percent of the circulating cells having this phenotype, again representing an expansion of dendritic cells in the peripheral blood.

Our first patient treated with flt3L, a patient with advanced melanoma at multiple sites, also developed rather profound vitiligo, as well as disappearance of a couple of subcutaneous lesions associated with FL treatment. This is

characteristic finding in patients responding to a variety of immunotherapies for melanoma, unfortunately, ultimately progressed with CNS metastasis.

We've also taken a group of patients and treated them with subsequent high dose IL-2. In a patient who had really what appeared to be stable disease these after flt3L, who had multiple subcutaneous and dermal and epidermal lesions here shown on the face of metastatic melanoma, as well as pulmonary nodules after a first cycle of IL-2 and a second cycle of IL-2 has had a complete disappearance of these lesions as well as the pulmonary lesions, consistent with the potential notion that some combination therapy designed to target both dendritic cells as well as
T-cells might be successful in such patients.

Now I'd like to take just the last few minutes and talk about delivery of tumor antigen into dendritic cells as opposed to driving dendritic cells into tumor. As was again shown from the laboratories of Nina Bhardwaj and shown again today by Ralph and Jacques, one can take a CD-86 positive dendritic cell, add in this instance di-I cultured melanoma cells and show within a few hours rapid

uptake into virtually all of the dendritic cells of these apoptotic bodies and apoptotic cells. In this instance representing serum starved melanoma cells, although we've done the same thing with irradiated cells, adeno P-53 transfected cells, as well as a variety of other strategies designed to drive apoptosis. These are studies that are relatively straightforward to perform. One can, in the green, express your CD-86 or DC marker and in this instance, red, use to stain your tumor cells and one can show a complete matchup of
CD-86 positive di-I positive cells, again consistent with rapid uptake of apoptotic bodies and apoptotic cells by these dendritic cells.

In addition, from our clinical trials where we're actually using GCSF stimulated peripheral blood, leuko phoresis-derived cells, one can demonstrate in some of these patients a failure to completely convert to a CD-14 negative phenotype. These represent adherent cells cultured in CMCSF in IL-4. You can see that there's a large number, even after five days of CD-14 positive cells that are also CD-86 positive. These, in our estimation, represent a mixed or a middle phenotype of

macrophages on the way to DCs, very similar to what's observed in CMV-infected cells. If you add, as was done in this patient, his irradiated renal cell tumor, one can demonstrate within a day to two days that you have driven the phenotype of these cells as a maturational step. Just by adding apoptotic irradiated tumor, one can demonstrate further maturation of these DCs just by the exogenous administration of irradiated tumor again suggesting that this might be a strategy to drive DC maturation as well as enhance antigen uptake. And this patient was treated repeatedly with this combination of irradiated tumor and DC preps.

We've done similar studies using apoptotic melanoma cells and again demonstrating that a GMCSF plus IL-4 cultured dendritic cell derived from adherence cell progenitors again when you add just overnight irradiated tumor here at a 10 to 1 DC to tumor ratio, one can demonstrate a rather pronounced increase in expression of this molecule CD-86.

Not only is one capable of doing that, but one also can use these tumor-fed DCs to promote

multi-epitope specific CTL. Shown here, one can demonstrate that one can elicit T-cells fully capable using the strategy to respond to the melanoma 526 as well as a number of peptide pulsed A2 presented targets, again demonstrating the power of using these DCs.

So I'd like to conclude and just thank some of the individuals involved with these studies. Michael Shurin and Clements Esche did many of the flt3L studies and Robbie Millard and let's see, who else should I pick out here?

(Laughter.)

My son who did some of the studies with apoptotic bodies. I should just finish and say that as many of you know, I used to be a T-cell chauvinist. I've now become a DC evangelist. I ran the Bordeaux marathon last September in France. They actually give you wine during the race. This is a picture of me at the middle of the race drinking a glass of nice French wine and they make you wear a costume. I went as Statue of Liberty and I really did it in honor of the dendrites on the crown of the --

(Laughter.)

Thank you for your attention.

DR. MULÉ: Thanks, Mike. Our next speaker is Dr. Drew Pardoll from the Johns Hopkins Oncology Center and it's a pleasure for Drew to share with us an overview of antigen-presenting cells and antigen processing in presentation in tumor immunity and tolerance to tumors.

DR. PARDOLL: While standing on one leg. What I want to start with before talking about antigen processing and presentation specifically is to just sort of define the problem, at least as we at Hopkins see it with regard to immunotherapy of cancer.

May I have the first slide? So it basically boils down to tolerance. And so what I would propose is that the paradigm that drove much of cancer immunology in the 1950s, namely the immune surveillance hypothesis which postulated that one of the normal roles of the immune system was to survey the body for tumors through recognition of neoantigens and to eliminate them based on this recognition is, in fact, incorrect, and needs, in fact, to be replaced with what I'll refer to as the immune tolerance hypothesis which essentially states that the response of the immune system to neoantigens that arise in tumors is more like the

response to self-antigens, namely, the natural response is tolerance rather than activation.

I'll show you just an example or two that this really is, in fact, the case. These are some transgenic studies done by Wadar Sodomayer and Hilavisky's lab in parallel with Adam Adler in my lab. The idea was to look at the immune response and sorry this is backwards, but it's okay. Hopefully, this will be the only one.

But in any case, the immune response to a model antigen hemagglutinin and expressed either as a self-antigen in a transgenic mouse expressing it off the C-3 promoter which expresses HA in the prostate and other parenchymal cells or expressing it as a tumor antigen on the A-20 tumor which one can inject into BALB/C mice. And HA, as you know, is when it comes into the body in the form of an antigen on influenza is a very potent antigen and immunogen and the question is what is it when it's a tumor antigen or a self-antigen and the system here, hard to see backwards is that one can transfer limited numbers of marked T-cell receptor transgenic T-cells into these animals with a transgenic T-cell receptor specific for an HA peptide in this case presented by MHC Class 2 molecules and then after

encountering the antigen in vivo in a setting where they represent a small enough proportion of the total T-cells, can be taken out of the animal and their functional state is determined.

What I'll tell you without showing you the data is that in all of the cases, namely HA is a self-antigen, as a tumor antigen or as a viral antigen, one can show by looking at activation markers on these transgenic T-cells that the T-cells recognized through their T-cell receptor HA peptide and also they're still present after that recognition.

In most cases they expand somewhat and then they contract back down, but they're certainly still present. So one can then pull them out and look at their function and hope that -- good.

So this is one of a number of assays that we used, the simplest being simply looking at proliferative response to the cognate hemagglutin peptide in vitro presented by good antigen presenting cells and what we're showing here is the response per clonotype positive T-cell. So this is really looking at the proliferative function per clonotype positive T-cell. What I'll tell you is

that if you look at lymphokine production one sees essentially the same thing.

So with HA as a self-antigen, what one see is not unexpectedly the induction of anergy, the now, probably most important form of peripheral tolerance, and so when these cells circulate in HA transgenic animals for one week, one loses about two thirds and then after two weeks about over three quarters of the responsiveness and this is the classic definition of anergy when HA is expressed as a parenchymal
self-antigen.

If now one looks at HA expressed on tumor cells as Eduardo did, even though a 20 which is a lymphoma cell that immunologists use for in vitro studies for antigen presentation, nonetheless, as a tumor antigen HA induces the same sort of anergic tolerance as it did when it was a parenchymal
self-antigen.

Compare that with the response when HA is a viral antigen such as this recombinant vaccinia HA here and what you see is the classic hyper responsiveness of a memory response of an activated cell.

So this is one of actually now a number of examples emerging in the literature to demonstrate that, in fact, the response of the immune system to antigens expressed by tumors tends to be that of tolerance. So the problem then is really breaking tolerance, taking either cells that have low affinity receptors and therefore are ignorant of the antigen and now raising them up to a state of therapeutically useful activation or potentially formally breaking tolerance by taking an anergic T-cell, rendered anergic, and converting it to an activated cell.

So we have to understand this process a little bit more and certainly one of the current driving paradigms is the model put forward by Bretcher and Cohen which is that applied to T-cells in this case which is that in order for a T-cell to be appropriately activated, it requires two signals, signal one being engagement of the T-cell receptor by the peptide MHC antigen, signal 2 being a co-stimulatory signal. We now know that signal 2 represents a host of different co-stimulatory signals. In the presence of signal 1, in the absence of signal 2 the default pathway would therefore be tolerance.

What we're now learning in the case of tumors as well as parenchymal self-antigens is that these decision processes, the APCs in all cases seem to be bone marrow-derived antigen-presenting cells. So what that means is that if tolerance is being induced, it's being induced because the antigen is being presented not by the original cell that expresses it necessarily, but rather by a tolerizing bone marrow-derived antigen-presenting cell. Likewise, if activation is the outcome, it's because it's a different kind or state of antigen-presenting cell that's providing the appropriate co-stimulatory molecules or signals in addition to signal 1.

And so one can then look at the response to tumor antigens versus antigens in a viral infection, really based on what the nature of the antigen-presenting cell is, the idea being that in the case of a viral infection, there are inflammatory or we'll use the term danger signals a la Polly Massinger which we now know to really involve a number of chemical species such as cytokines, such as GMCSF and TNF alpha which activate antigen-presenting cells and that results in the appropriate signal delivery to

T-cells. In the case of tumor, there do not exist the appropriate danger signals and so the outcome is tolerant.

So in essence, what all of cancer vaccines probably are trying to do is two things. One is to deliver reasonable doses of the right antigens to antigen-presenting cells and the other is to create the appropriate danger signals that will activate antigen-presenting cells into this activation mode which we now refer to as the dendritic cell.

In our own case, we found that a vaccination approach that we had been using, namely to introduce cytokine genes into tumor cells and vaccinate with these cytokine gene transduced tumor cells turns out probably to be an approach that generates dendritic cells in vivo local to the site of antigen. We didn't really know we were doing this at the time of the experiments which were done in collaboration with Glenn Drenoff and Rich Mulligan in which we empirically looked at the ability of a poorly immunogenic tumor, the B-16 melanoma transduced with a whole host of different cytokine genes to induce protective immunity shown as the ability to reject tumors up here and what

fell out in this very empiric, totally nonmechanistic study was that GMCSF transduced tumors work the best and it was needless to say extremely exciting and revealing to us when we were seeing these sorts of results to begin to see the results from Ralph Steinman's and others' laboratories demonstrating the importance of GMCSF in generating the differentiating signals towards progenitors towards dendritic cells.

So the idea then with these GMCSF transduced vaccines is that the transduced vaccine was a depot for locally for tumor antigen as well as GMCSF and in the presence of this local GMCSF, bone marrow-derived progenitors were induced to differentiate to antigen-presenting cells. One can show with all the markers that in addition to macrophages and everything else that there are dendritic cell marker expressing APCs that abound within the vaccine site and they eventually appear in draining lymph nodes.

What we also were able to demonstrate with model antigens in the tumor using chimeric experiments, these are experiments done by Paul Lumback and Alex Wong, was that when antigen is taken up into these antigen-presenting cells in

vivo, it's not only processed into the MHC Class 2 pathway, but also is efficiently processed into the MHC Class 1 pathway, this phenomenon known as cross priming. And in studies using bone marrow chimeras in which bone marrow donors were mice that were genetically knocked out for various molecules or genes and coding molecules in the Class 1 antigen-presenting pathway, it's now quite clear that this cross priming pathway for the presentation of Class 1 antigens goes through the cytosolic pathway, so that if one eliminates a tap in the bone marrow-derived cells or if one eliminates LMP-2 or LMP-7 looking at an antigen that is LMP-2 or LMP-7 dependent for proteosomal processing, one loses this cross priming.

So I think one of the very important areas of investigation now for vaccine development needs to be understanding exactly how it is at these antigen-presenting cells cross out of -- will cross antigens out of the endosomal compartment and into the cytosole.

We thought that to further develop or understand this, we needed to have an in vitro system in which we could demonstrate that antigens delivered to, for example, dendritic cells in vitro

exogenously could be crossed into the Class 1 pathway. And even though a number of investigators such as Ken Rock have shown that really ridiculously super-physiologic concentrations of antigen could achieve this, it turns out to be very difficult to actually see this with normal amounts of antigen, but we were encouraged, certainly by Nina Bhardwaj's studies demonstrating that antigens in apoptosing cells could, in fact, in vitro be taken up by dendritic cells and presented by MHC Class 1 antigens.

Pursuing this, one of the graduate students in the lab, Jianglin Lu, using the HA system and dendritic cells, followed this up further and in his hands he found that when one simply adds HA in the form of lysate of a tumor cell expressing HA to dendritic cells and then adds CD-8 positive T-cells specific for HA, there's really very little activation of these cells.

It wasn't until he added cognate helper cells to the dendritic cells shown here, so these are adding helper cells specific for HA, that he was, in fact, able to now at quite reasonable concentrations or doses of antigens, generate this in vitro cross priming with dendritic cells. It

could be partially replaced by simply infecting the dendritic cells with a vaccinia virus indicating that this infection generated some internal signals which activated this cross priming pathway.

Interestingly, in contrast to what one might have expected from some of the in vivo studies, an agonist, anti-CD-40 antibody failed to replace helper cells themselves in allowing this cross priming to occur.

Just to give you an example of the potency with which one can see this cross priming, this is simply looking at the CD-8 response when tumor lysate is diluted down towards 1 to 1,000. And of course, when you do that and have CD-4s and CD-8s present, what you see is this drop off, but this drop off could be from a decrease in presentation on the Class 1 site or the Class 2 site. However, one can separate the systems and add simply the Class 2 peptide at the phase in which the helper cells are added to the dendritic cells and what you can see is that in this case the helper cells sensitize the dendritic cells in a way that now even very, very small amounts of antigen in the form of diluted tumor lysate can still, nonetheless,

cross prime in the sense of be processed into the MHC Class 1 pathway.

Notice here that in the absence of helper cells, one does begin to see a little bit when with high amounts of lysate and in fact, when one concentrates the lysate further, one can show that again at very large amounts of antigen one can do away with helper cells. However, with the levels of antigen that we think represent physiologic levels, the importance of having cognate help to sensitize dendritic cells we think is going to turn out to be really quite crucial.

In fact, helper cells which had been ignored completely by the cancer immunology community for many decades are really undergoing a revival and I just want to show you an example of some studies that Ken Hung did with the GMCSF transduced B-16 vaccines to demonstrate the importance of helper cells in mediating a number of additional effector pathways besides simply activating CTL and you can demonstrate this in knockout mice in which one can see that relative to the protection generated in wild type mice, there is, as expected a loss in protection in CD-8

knockout mice when one vaccinates with GMCSF transduced tumors and then challenges.

However, there is still a reasonable level of protection. Instead if one vaccinates a CD-4 knockout mouse, one loses all of the protection, so the difference between basically this line here and this line here, represents CD-4 dependent anti-tumor effector mechanisms that are other than CD-8. And in fact, if one simply looks histologically in the challenge site of a wild type animal vaccinated with

-- I don't know if we can focus that, vaccinated with B-16 GMCSF cells, one can, in fact, see an infiltrate of eosinophils that is lost in the CD-4 knockout mice. You'll have to take my word for it, since they don't seem to be focusing this too well.

In CD-8 knockout mice, there's really no difference.

So it turns out that those eosinophils are a TH-2 dependent effector. In fact, there's also important TH-1 dependent effector that is independent of CD-8s and that turns out to be macrophage-dependent INOS and what you can see here is that INOS is induced quite dramatically within macrophages infiltrating a tumor challenged site in

animals vaccinated with B-16 GMCSF vaccines. Again, this is completely CD-4 dependent.

What I won't show you is that this is a TH-1 dependent phenomenon and that in INOS knockout mice one loses about 50 percent of vaccination potency as does one lose about 50 to 60 percent of vaccination potency in GP-91 knockout mice which are -- which is one of the subunits of the NADPH oxidase complex responsible for superoxide generation.

So I'll leave this actually as -- how much time do I have? I'm out. Okay.

(Laughter.)

Jim is so intimidating. So this will be my last slide. So I simply want to point out here and this certainly has a lot to do with issues of what one assays for in attempting to determine whether one's vaccine is really doing what it's supposed to be doing. The point here being then that through this, in our case, in vivo dendritic cell generation mechanism, one generates in addition to CD-8 positive CTL through helper cell sensitized dendritic cells in the lymph node, an additional set of effectors that is CD-4 dependent, that in fact, in this particular system generates both TH-1 and TH-2 effectors. The TH-1 effectors activating

macrophages that have picked up tumor antigen from the metastatic deposit presented on Class 2 and then these activated macrophages produced both nitric oxide as well as superoxides and probably other interesting reactive oxygen intermediates as well where as the TH-2 pathway activates eosinophils which turn out to degranulate and eosinophil granules contain a number of very potent cytocidal molecules.

What I haven't shown here is antibodies, although antibodies probably also support this response by oxidizing tumor antigens to amplify this cycle through MAC Class 2 positive cells at the site of metastatic tumor.

So in fact, I think the take home message is that with the appropriate delivery of antigen and activation of antigen-presenting cells, there may be a number of important immunologic effectors that one needs to think about in terms of being brought to bear in a synergistic fashion to generate the most effective anti-tumor immunity.

(Applause.)

DR. MULÉ: Thank you. Our final speaker of this session before we have our panel discussion is Dr. Larry Kwak from the NCI, keeping in line with

what Drew had introduced with respect to GMCSF. We'll hear a bit more about that in Dr. Kwak's talk which is recruitment of dendritic cells for tumor antigen processing for T-cell recognition in vitro and in vivo.

DR. KWAK: Actually Drew -- could I have Drew's last slide? I'm going to start with that.

(Laughter.)

I think you'll find that many of the observations that I'm going to share today actually impact on several of the topics that are being raised by this symposium, including some already discussed today, as well as those tomorrow and the current session on dendritic cells.

The studies that I'm going to share with you today are focused on the study of an antigen called idiotype which we have been focused on for about a decade now. The concept of the -- I think my pointer went dead.

The concept of the -- as shown on this slide and it's that the immunoglobulin receptor, the antigen receptor on a malignant B cell is an immunoglobulin molecule. The clonal proliferation of tumor cells from the neoplastic clone will be unique from all other B cells in the human body in

that there will be a unique constellation of amino acid sequences generated by the heavy and light chain genes. This particular -- this is a slide actually that was taken from a publication by George and Frieda Stevenson in 1971 who were the first really to propose idiotype as a tumor specific antigen for B cell malignancies.

As shown on this slide is the spectrum of normal B cell differentiation and some of the malignancies, the spectrum of malignancies that could be envisioned for targeting of this receptor by vaccine approach, including CLL, B-cell lymphoma and multiple myeloma.

The pre-clinical rationale, the seminal experiments for this approach were actually also done in the very early 1970s in the laboratory of Eisen, who first demonstrated that an immunoglobulin derived from a MOPC plasma cytoma could be used to immunize syngeneic mice in such a way as to raise an antibody response that was specific for that idiotype portion or the variable region portion of the immunoglobulin. These mice could subsequently be protected against tumor challenge against the particular tumor from which the immunoglobulin was

derived and that this tumor resistance was also specific for the idiotype portion on the molecule.

Since that time, this basic phenomenon of idiotype specific tumor resistance has been reproduced in a number of lymphoma myeloma and leukemia models and so it's on quite firm basis that we stand for the clinical trials that I'm going to spend the majority of my time describing to you.

Now the very first human clinical trial was done by myself working in Ron Levey's laboratory at the time in which we immunized nine patients with B-cell lymphoma with immunoglobulin derived from their own tumors. This was an important study, but it was a pilot study, just the beginning because it showed largely that one couldn't raise an antibody response similar to the animal experiments of Eisen several decades ago, but largely what was missing were T-cell responses in these patients and this was a heterogeneous group of patients and the question of clinical efficacy was not to be addressed here.

Subsequently one established the program at the NCI. We established several goals, two of which are shown here for subsequent human lymphoma vaccine development. The first was to make further improvements in vaccine potency so that we're able

now to systematically assess the question of anti-tumor efficacy and I'll show you how we've done that in the current clinical trial, but secondly, to complete the answer of immunogenicity, by focusing on the generation of CD-8 positive T-cell responses.

This is the schema for the current clinical trial. It's a single arm protocol and it has several distinguishing features which I'll go through briefly. The first is that because vaccination is going to be -- is going to depend -- the success of vaccination is going to depend on the ability of the host to mount an immune response in this disease particularly follicular or low-grade lymphoma, we felt justified in going into previously untreated patients. These patients are all after a lymph node biopsy to obtain starting material for the vaccine, are uniformly treated with the same chemotherapy regimen. The particular regimen is probably not important. What is important is that at the end of this chemotherapy, that one has a homogenous group of patients who are in their first clinical remission from their disease. After a six to 12 month waiting period after chemotherapy, the series of vaccinations is given with the autologous idiotype, derived from the patient's own tumor,

coupled with KLH as an immunogenetic carrier and then as Drew laid the groundwork for the use of GMCSF, in this case, soluble GMCSF mixed with the antigen and administered over a total of four days.

So in this first part of the talk one can think of this representing a recruitment of dendritic cells in vivo and those are the immunologic and molecular results that I'm going to share with you. But before I do that, I want to make the point that in the field of cancer vaccines unlike that of chemotherapy where the final question is that everyone wants to get to is can this therapy produce clinical benefit, we have to answer this first question -- this other question first and that is, is it even possible to immunize against the self-tumor antigen?

Now in trying to answer that first question of whether it's even possible to immunize against the tumor antigen, we spent quite a bit of time in our laboratory thinking about what the most convincing level of evidence would be to show that we've actually accomplished immunization.

These are what I've listed, these two are targets that have been used traditionally for measurement as targets or the T-cell response in

cases where the antigen or the peptide is known, autologous antigen-presenting cells pulsed with those peptides and antigens have been used. Even better, when available, allogeneic tumor cell lines have been used as targets. But we felt that the very best level of

-- most convincing level of evidence would be obtained by using autologous, in each case using autologous tumor cells. And so all of our assays have now been adapted towards the use of autologous tumor cells as targets and admittedly, working with hematologic malignancies, it's somewhat easier than working with solid tumors, but it's a human experiment that nevertheless needed to be done.

Twenty patients who were in complete remission, first complete remission on that clinical study have completed vaccination and those are the results that I'm going to share with you today. These are still unpublished and were the subject of a plenary session presentation at the American Society of Hematology just three days ago.

The first question of T-cell immunity to this antigen has been addressed by Maurizio Bendandi, who is a Fellow in the laboratory who simply took PBL from immunized patients and put them

in culture with autologous lymphoma cells, follicular lymphoma cells in each case and asked the question of whether the -- after five days asked which cytokines were being made in response to that stimulation. And what's shown here are impressive levels of TNF production. Also consistently observed are GMCSF and interferon gamma and basically the bottom line is that 17 out of these 20 patients make specific responses to their autologous tumor. Now in the interest of time I'm going to show you the controls that you undoubtedly are asking for on the next few slides.

These are two representative patients which show first that tumor specificity of the response as shown by the lack of response of immune PBMC for normal flow sorted peripheral blood B cells as nonneoplastic targets. Secondly, what this experiment shows is that the stimulation of this response against autologous tumor can be blocked by the addition of an anti-Class 1, but not by an isotype control antibody or anti-Class 2.

When we asked the question of whether these T-cells, they can make cytokines, but can they actually kill the autologous tumor cell in vitro? We had to do one additional manipulation and that's

shown on the left part of the slide in blue and that is to prestimulate the stimulating tumor cells through the CD-40 receptor with fibroblast cells which are transfected with the CD-40 ligand. When those tumor cells, prestimulated tumor cells are used in the assay I just described, that is, taking immune PBL, stimulated with tumor cells for five days and then assayed on unmodified native tumor targets the following results were obtained. In the vast majority of these patients that I've shown you, two representative for simplicity here, we observed significant -- substantial amounts of killing of the autologous tumor cells in each case that was evident post-vaccination, but not pre-vaccination. As controls for specificity there was lack of killing on non-neoplastic normal B cells and when available, EBV lymphhoblastoid lines from these very same patients to demonstrate the tumor specificity of these T-cells.

The lysis of these autologous tumor cells was also blocked by the addition of anti-Class 1 pretreatment and not by control antibodies and when he fractionated out the CD-8 positive T-cells, these were sufficient to mediate that level of lysis. Now in terms of trying to answer that second

question, trying to begin to answer that second question of anti-tumor effects, the difficulty in the design of the trial is shown here and that is that all of these patients by design are in a minimal residual disease state, complete remission at the time we give vaccinations, so that standard tumor shrinkage criteria cannot be used to ask whether the vaccine had an anti-tumor effect.

However, there is a molecular surrogate marker for minimal residual disease in this particular disease and while we're not saying this is good enough for licensing of a drug, to prove scientific -- proof of concept, I think you'll agree it certainly is valid. And the premise is based on the fact that even though all of these patients who are in complete clinical remission have no disease by standard clinical criteria, they will all be PCR positive in the blood or bone marrow by using this PCR technique.

And the PCR reaction, the molecular marker is of course the 1418 translocation which involves the rearrangement of the BCL-2 oncogene. This rearrangement is detectable by PCR in about 50 to 60 percent of patients with follicular lymphoma and again, I don't have the time to go through the

entire details of the analysis, but I'll just try and hit the high points. It's important in doing an analysis like this that it be set up properly and I think we've accomplished that. These assays were all performed by individuals who were blinded to clinical information. The assays were -- each sample was analyzed by individuals who were blinded to the information at two independent sites here at the NCI as well as with a collaborator at Penn State, Hershey, and 10 replicates were used per time point, internal controls and in each case the rearrangement was -- the amplified product was confirmed by nucleotide sequencing. And 11 of those 20 patients had tumors which were -- that had break points that were amplifiable by this nested PCR reaction. Eight of those patients converted to PCR negativity after the vaccine while three others remained persistently PCR positive in the peripheral blood and these are ten replicates at each given time point.

This is a summary of the PCR results on those 11 patients. Again, 8 out of the 11 converted from PCR positivity pre-vaccine. All of them were PCR positive, even though they were in a clinical complete remission. Eight converted in the red

boxes to PCR negativity and have maintained this PCR negative status at serial sampling for up to 27 plus months continuously. Of the three patients who were PCR -- who did not clear detectable disease, one of them has relapsed and is one of the two patients out of the entire group of 20 who have relapsed, the remainder remain in first continuous complete remission.

And so in wrapping up this first part of the talk, these first two points correspond to the two questions that I posed at the beginning and that is the first question is whether we could even immunize against this particular antigen. We believe through some of the data that I've shown you today, that we're arriving rapidly at the answer in the affirmative for this particular antigen and that's demonstrated by 17 of 20 patients vaccination against idiotype has elicited the first evidence for CD-8 positive T-cells specific for their autologous tumor cells.

These data are also important, perhaps equally so, because they formally suggest that follicular lymphoma cells can present the endogenous idiotype presumably as peptide MHC complex to CD-8

positive cells and therefore can serve as targets of immune response in vivo.

Secondly, in terms of anti-tumor effects, aside from -- well, 8 out of 11 patients whose tumors were mbr positive converted to PCR negativity, we believe providing the first systematic evidence for an anti-tumor effect of idiotype vaccination in this disease, aside from several anecdotal reports that have been published of isolated regressions of tumor masses in patients who are already in a minimal residual disease state.

We think that the reason that we're seeing both of these effects, profound immunologic effects, as well as the very provocative molecular responses so easily is the GMCSF which we think is a critical factor of this particular vaccine formulation. And these are just the clinical, just in writing, the clinical results, the clinical outcome of these patients that 18 of 20 remain in continuous first CR with a median follow up of 36 plus months after the completion of induction of chemotherapy. Of course, what's needed to provide the final answer to that second question now is a randomized multi-center controlled trial to vaccine versus not.

I'm going to just use the remainder of my time to illustrate an approach in which we are exploring the use of dendritic cells in vitro with this particular antigen. This slide illustrates an alternative strategy that we have been pursuing for some time now. This is actually the clinical history of the first patient who was published in Lancet in 1995 in which this is really an adoptive T-cell immuno-therapy approach in which we've actually immunized the donor, the healthy marrow transplant donor and achieve transfer of T-cell immunity from donor to recipient through the bone marrow innoculum.

For all the reasons that we might have -- that you've heard today that we might have difficulty immunizing the autologous tumor-bearing host, these are the reasons that we should be trying to immunize normal donors or cells derived from normal donors for adoptive transfer. And I'll just point out the last one that from a cancer vaccine standpoint, it's been particularly appealing to us because immunization of a healthy donor should be relatively easily accomplished compared with the tumor-bearing patient.

Well, since that time we've evolved our thinking some and there are probably inocula, transfer inocula that are more effective than bone marrow for transfer of T-cell immunity if what one is trying to really transfer ultimately is essentially a pure population of tumor specific T-cells and this stands for donor lymphocyte infusion for those who are not in the hematology field.

And secondly, for various reasons it's probably appealing to be able to do this whole thing, maneuver this whole immunization of donor T-cells in vitro rather than or in addition to in vivo. And the approach that's shown here is as one could guess is to take donor T-cells and to do a primary in vitro immunization using dendritic cells, to expand those

T-cells and to give them then, subsequently transfer them to the patient as a tumor-specific T-cell immunotherapy. And in collaboration with Kim Lierly and Yee Wen Lee and their laboratories at Duke, we have accomplished this in a pilot study of a single patient in whom we've accomplished the top part which I'll share with you in the next two slides, that is the primary in vitro immunization for T-cell immunity.

Yee Wen Lee took peripheral blood from a leukophoresis from a normal donor and took the adherent cells and established isolated dendritic cells with GM and IL-4 and pulsed those using those dendritic cells that were pulsed with the whole immunoglobulin protein derived from the myeloma of the recipient, then sensitized T-cells through multiple rounds of stimulation. And this T-cell line now, the activity of this T-cell line is shown in this slide and specifically for its activity against the autologous, for its ability to kill the autologous, that is the recipient plasma cytoma cells. So again, we're using the autologous tumor cells as the target here for read out.

What he has shown is that these T-cells are capable of killing the autologous plasma cytoma target and again in a tumor specific manner because normal B cells from the same recipient are not killed and that this killing can be blocked again by anti-Class 1.

There are both CD-4, this line consists of both CD-4 and CD-8 positive tumor cells. They make substantial amounts of interferon gamma. This is a fast immune assay and other type TH-1 type cytokines in response to the autologous tumor cells

stimulation and again this response is tumor specific.

So I'd like to just close with this final slide which illustrates several aspects that we haven't been able to share with you, but that is the exciting ability to develop other formulations of this particular antigen, including those such as chemokines fused to the antigen of interest which probably also work by activating, recruiting dendritic cells in vivo. I've shared with you how we've used dendritic cells to generate T-cell immunity in vitro and lastly, one of the most exciting aspects is the existing clinical trials, taking material from those trials back into the laboratory to answer basic questions in immunology.

This final slide is perhaps most important. It's an acknowledgement of the individuals from my laboratory who performed much of the work I presented. I want to acknowledge also Craig Reynolds and Jay Greenblatt for their help, continuing help with production aspects in support of the clinical trial as well as regulatory aspects and somebody once told me that you know you really have arrived when you have your own beer. Thank you.

(Applause.)

DR. MULÉ: Thanks, Larry. I'll ask now all the session speakers to come up front and we'll spend some time going over some issues in a panel discussion. On behalf of Dr. Steinman, myself, I'd like to thank all the speakers for their presentations. So if we can have everyone come up front here.

DR. MARTI: I too, would like to thank all of the speakers of this afternoon's session for a tremendous amount of information that they've shared and the battery on my watch is dead, so please, somebody watch the time. I think there's a Medicine Review Board course in this room at 5 o'clock. Anyway, on page 14 of your program book are the questions that have been formulated for the panel and I'm going to just start with the first question because I think it's been very central to many of the presentations this afternoon. And that question is what is the immunophenotype of a dendritic cell? Can we have a common immunophenotype that can be agreed upon in the form of consensus, a single tube assay? Would it be two color, three color or four color? And also, while the panelists are thinking about identifying the

dendritic cell, I think that it's just important, as important, to think about the other cells that are in that preparation. And again, this is not so much for the idea of purity, but it's to have some feeling for what's in the product and what's being infused into patients.

So perhaps, Dr. Steinman, we can start with you.

DR. STEINMAN: Yes, I think the point is product characterization and I think the useful markers would be the lineage markers, CD-3, CD-14, CD-19, CD-56 and so the dendritic cells shouldn't have those markers. And then you want DR and I think you need to know the level of co-stimulators. The three that we like the most are CD-40, 54 and 86.

And then you get to the dendritic cell restricted markers, so it's currently available are 83, hopefully DC-LAMP will come soon. So think that's a pretty big panel. That will cost you $5,000 probably.

(Laughter.)

DR. MARTI: What about ILT-3?

DR. STEINMAN: The ILT-3, my understanding of that family is that it's broadly

expressed on myeloid cells and I think you were taking advantage of other scatter properties in getting the specificity that you did. I think after culture, especially,

ILT-3 will be on many different kinds of myeloid cells, but it's a little early there.

And then I just want to emphasize two things you brought up, Jerry, which is one that we've got to standardize our facts and you gave us terrific leads with those issues of cytometry and the beads, and secondly, that we've got to start using permeabilized specimens. For example, CD-83 is found inside the immature dendritic cell and it's maybe a very helpful marker for that state. DEC-205, human equivalents are just coming in, but that's largely intracellular, it's elect and receptor on the dendritic cell and DC-LAMP, of course, is almost entirely intracellular. So I think that's going to be informative.

DR. MARTI: Thank you. Mike?

DR. LOTZE: All of the studies in terms of characterizing DCs in our clinical protocols have been worked out in our immunologic monitoring and diagnostic labs by Elaine Elder and Tracey Whiteside and in addition to the ones that Ralph talked about

where we do a cocktail of antibodies to define lineage negative cells against CD-86 or Class 2, we also increasingly have been using the GMCSF receptor alpha chain and the IL-3 receptor alpha chain to differentiate the lymphoid, I should put nominally lymphoid dendritic cells and myeloid DCs. You can't really define cell of origin based on just phenotype.

I also think it's important, the point that was made by Ralph about the contamination by other cells. Many of the results that I see coming out of our clinical trials gate on the large cells and you tend to ignore the fact that there are a large number of contaminating other cells, most of which are T-cells which cluster with the DCs very effectively. And so since we know as Drew emphasized earlier and I think Jacques, these T-cells can also modulate DC function, both in culture and presumably after adoptive transfer. It's important we try and get a handle on this nominal T-cell contamination.

And then I also think -- just as one final point, is I think the issues related to maturity of DCs is again an issue which over and over again has been thematic for the last couple of

years where the bright expression of Class 2 CD-86, CD-80 and the mature DC marker, CD-83 as well as P-55 active bundling protein are the ones that we're going to have to add to define the DCs that we're giving. I'm hopeful at the end of the next couple of years of clinical testing that we'll be able to say these are DCs that were derived that had some kind of clinical endpoint if not clinical endpoint antigen response endpoints which ultimately are going to be the things we'll dictate what the best DC is for clinical trials.

DR. MARTI: Dr. Banchereau, I'm not so sure that you agree that there is T-cell in your preparations. What markers would you suggest for the other non-dendritic cells?

DR. BANCHEREAU: First, one needs to know what he or she wants in terms of the phenotype of DC. I mean it's an important question. Do we want immature cells? Do we want partially mature cells? Do we want to fully mature cells? That's the number one question.

I don't think anyone has addressed that yet. So that's going to be dictating the phenotypes you want to look at. Certainly what we heard from

Ralph and Mike we fully agree with. How to use it, that's another question.

The second question is the subpopulations. Do we want to Langerhans cell types, do we want interstitial DC type. Again, we have specific markers for that. We know that the CD-2 is expressed on the interstitial DC, CD-9 expressed on interstitial DC. We have LAG expressed on the nonligand cells. Those may be a refinement, that we may want to go for because according to what we are searching, we may be interested in knowing that. Finally, the contaminants, the contaminants are important. We don't have much T-cell contaminants using the C-34. I think the people doing the monocytes have more of that problem.

Now, is that a real contaminant, is that a beneficial contaminant? Is that a nonbeneficial contaminant? Who knows? Again, those are questions to address and certainly in the case where you do CD-34, one contaminant you should pay attention to are the basophil are eosinophil. If you don't do a Giemsa and if you -- you may be biased by your facts because again, the facts don't show you what cell type you have, so I have forever been pledging for people to do a Giemsa staining to see what cell type

do you have in your culture because it avoids the gating which is very dangerous. And for CD-34 cells, we do find TNF to be very essential to avoid the development of basophils and eosinophil. Now, a few basophils and eosinophils, I don't think is going to hurt you, but we had been playing with some population where we are setting up all the technology for the application to the clinical trials. We had in some cases a lot of eosinophils and basophils. And I don't think you want to inject that in too large quantities.

So those are the considerations. Don't forget the Giemsa.

DR. MARTI: Would any of the other panelists care to comment further upon immunophenotyping of dendritic cells before moving on?

DR. KWAK: Well, I think there are others in the audience, especially, I might ask Kim Lierly to comment on your dendritic cell preparations? Maybe you're going to talk about that tomorrow.

DR. MARTI: Is she here? He, I'm sorry, thank you. There's a microphone, at least one I see here.

Perhaps it will come up again. If there are no further comments on the --

DR. MULÉ: I would like to raise one issue which is in respect to if we agree on a panel of antibodies that one would use to characterize DC for the clinical trials and whether that's a panel to be used for CD-34 derived versus monocyte derived, I think one thing we also have to keep in mind is that there is significant variability in patients with cancer with respect to the profiles that one sees when one standardizes the culture conditions for dendritic cell generation. It's predicated on the previous treatments that the patients have received, the tumor type and another good example is in CD-34 mobilization strategies with GCSF, the types of cells that are immobilized depend critically on the mobilization strategy that's employed and a good example are tumor cells that may also be mobilized to the peripheral blood when one uses GCSF. So it's not a simple concept of just using a defined panel of antibodies, but it's more complicated when one looks at the clinical situation in cancer patients.

DR. ESSAYAN: Is there any utility to an algorithmic or order of different markers. Can you

reduce the $5,000 aspect by looking at more expeditious methods of identification of dendritic cells?

DR. STEINMAN: You know, we just look down the microscope and we know what we have, but I just think we're at the stage where we're using different sources and different labs and we just won't be able to standardize unless we all collect the data. The issue is whether the NCI can help us and start providing these antibodies, especially some of these ones that are getting a little hard to get.

DR. LOTZE: I like the idea of antibodies to gamma interferon for the LE spot. That would be a good thing for the NCI to provide.

(Laughter.)

The one thing that I would argue is that I think a premature rush to standardization to define virtue in the absence of crisp, clinical endpoints may be a bit premature in the sense that we're going to have to go through a period of exploration before we can come to conclusions and I think we ought to use the best available markers and the best available information today, but we ought to not rush to judgment as to which cell is the best

cell or the best phenotype before we collect what is really the endpoint data, ability to generate an effective immune response, either to nominal antigens or particularly to tumor antigens and the associated clinical response.

DR. MARTI: I think Dr. Stewart has a question.

DR. STEWART: I just wanted to make a comment that I agree with what Mike just said, if we rush into this too fast we may come to the wrong rules, let's say. We seem to like rules nowadays. But at Roswell, we have created 12 different combinations of antibodies and I just noticed four more that I didn't realize were important in DCs and getting normal reference ranges of DCs with the strategy that basically everyone is using, but I'm trying to take every antibody that's ever been reported on a DC to be as comprehensive as possible to develop a normal reference range in a normal population from which we can now base therapeutic or clinical effects of our treatments and we hope to have that study done in the next three or four months and I don't think it's going to cost us $5,000.

DR. LOTZE: Carleton, are you doing that for what's in normal peripheral blood or based on what's in cultured cells. Part of the problem is once you start manipulating cells in culture and subjecting them to a variety of different environmental milieu ex vivo, then you've got a zillion different phenotypes.

DR. STEWART: Yes. Right now, we're just focusing on what you're starting with and presumably what you would have in the patient after you have done something.

We have four groups at Roswell who are working on DCs and I know who gets the best populations and who gets the worst ones.

DR. LOTZE: Best and worst is judgmental and based on what?

(Laughter.)

Based on clogging your flow cytometer or?

(Laughter.)

DR. MARTI: You know the question that was asked not only at baseline, what goes into the culture, but in reference to what is in the culture at the end that you're going to infuse. Do the investigators have any limits? I mean, if you only

have one percent, do you still go ahead and infuse that; or if you have 10 percent, do you infuse that? Where do you not infuse?

DR. STEWART: I think this was brought up all throughout all of your talks, the fact that we have a heterogeneous group of laboratories studying a heterogeneous group of cells, and even in our own institute we can see that different people are isolating their cells in different ways, culturing them in different ways, and because they all -- I provide the flow for all of them, I can see the differences and I know what you're talking about. And there's not going to be any consensus until we all figure out what's going on, because maybe everybody is doing it right for what they're doing. The problem is they don't know what they're doing.

(Laughter.)

DR. MARTI: Would you go to the microphone? This meeting is being transcribed and perhaps state your name.

DR. WEBER: It's Jeff Weber from USC. Just to address Carleton's point, I would think the gold standard should still be in the clinical trials whether you see clinical benefit. To address your

question of whether you should infuse DC if it's 10 percent or 25 percent or 50 or 80, if you can see clinical benefit, i.e., shrinkage of disease or what ever other surrogate immunologic marker you use, maybe it should be 10 percent, but I wouldn't make arbitrary assumptions until the data are in, and they probably will not be in for a year or maybe two years.

DR. BANCHEREAU: The only problem actually is that you want to know how many DC or bona fide DC you have injected. I mean whether you have to inject 10 million cells, total cells, to give you one million DC or whether you just inject one million of pure DC, one of the problems is to know whether your 9 million contaminants would not be limiting the effect of the DCs. I think that's the issue.

DR. LOTZE: Can I bring up one other issue? Everything we've talked about right now is phenotype and at the same time I think functional assays which have not been discussed so far by this panel, including ability to opsonize FITC latex beads, the opportunity to stimulate in the mixed lymphocyte response where I think Ralph showed some data with your perfect T-cell donor who responds to

all of your DCs, the ability to make the dendrokines that we can identify, IL-12, alpha interferon, are also other potential assays are important. Phenotypically, we see a similar set of DCs from hepatitis C patients and yet if you look at their ability to make IL-12, they are functionally deficient and yet phenotypically they are not dissimilar from the other DCs we get from other individuals, and so I think some functional assays might also be in order as you assess DCs.

DR. MARTI: You know this was a big problem with CD-34 cells, trying to find a functional assay that didn't take 7 to 10 days or 5 weeks for cobblestone assay.

We did have one speaker this afternoon, Dr. Urdal who raised a very provocative question about the use of CD-54 as a -- I guess an activation marker, something like CD-69 on T-cells. Does the panel, or Dr. Urdal like to comment further on that?

DR. URDAL: Yes, only to make the point that it is clearly CD-54 is one of those markers that I completely concur is upregulated in the system that we work with. Most people would cite it as a marker that's useful to quantify numbers of dendritic cells you might have in the culture; and I

think it's coupled obviously with biology, that if you inhibit that molecule it's clearly required for interaction with naive T-cells in the presence of an antigen for antigen presentation so it's at least linked to the biology of that interaction.

DR. MARTI: Do any of the other panelists have experience with flow cytometric-based functional assays or markers?

DR. LOTZE: We're doing some assays in our own laboratory where you use permeabilized DCs with staining of alpha interferon and IL-12 after stimulation with SAC or LPS gamma interferon as a way of defining at least their IL-12 production capabilities and that's a relatively brief assay.

The other one which has traditionally been used is just the ability to uptake particular antigens where you could use a variety of ones coupled with a fluorochrome like FITC.

DR. BANCHEREAU: But that would not work if you want to inject a mature DC, because a mature DC is not phagocytosing.

DR. LOTZE: And again, this will distinguish that functional characteristic of an immature versus a mature and can be used both in the negative as well as the positive.

DR. MARTI: If there are no further comments either from the audience or from the panel, we'll --

DR. KWAK: I've got one comment.

DR. MARTI: Go ahead.

DR. KWAK: Dr. Weber made a statement that I think needs to be challenged. I really disagree that clinical endpoints are going to be able to guide you with subsequent development of your clinical trials. Unless you're dealing with a very homogeneous group of patients, you're never going to make any sense out of those few anecdotal clinical responses or the majority of patients who don't respond. I think you really have to answer that first question first which is can you make an immune response. You pick an endpoint that you believe in and use that to monitor and guide your subsequent modification of clinical trials.

DR. KUFE: We heard this morning that acceptable endpoints were clinical responses but not immunologic assays. I think that needs to be clarified.

DR. KWAK: I guess that's for -- the FDA folks can speak for themselves, but I think that was for licensing considerations, not for doing science.

DR. MARTI: Perhaps this might be a good transition point to Question 4 which deals with functional assays and I know that in the remaining 15.5 minutes that we have in this auditorium that we won't get -- arrive at any consensus, but is it possible between the types of assays with regards to proliferation of cytotoxicity and cytokine secretion is the most important thing to show some type of antigen specificity? And don't everybody jump in at once.

DR. LOTZE: Maybe I'll just comment about the melanoma as a perfect place to start, perhaps idiotype and lymphoma where you actually have a defined antigen. Much of the problem with many of the other nonantigen, specific driven approaches is you don't have a target. So I think clearly if you're immunizing with peptide X, mart 1, GP-100, tyrosinase, TRP-1, whatever it is, that it would be not novel or amazing to think that T-cell assays in response to that are an important part of your immunologic monitoring. I guess the critical question in our mind is where you look for such cells. Traditionally, they have been looked for in the peripheral blood. Our approach has been to increasingly look at the site of tumor or at a

surrogate for tumor, DTH site, which we biopsy and then look at those T-cells as potentially a way to look for specific T-cell responses.

DR. MARTI: Before the panel continues, Dr. Puri reminded me that we also in terms of the functional assessment are concerned about whether or not we can measure or should measure quantitation of the peptide load. I think Dr. Banchereau, I really liked his cartoon drawings that showed in a very graphic way the loading of the dendritic cells. I know that we're going to hear some more about this in the conference, but in addition to proliferation, cytotoxicity, and cytokine secretion, when the ability to measure antigen-loaded cells, would that be a better one? And maybe, Dr. Banchereau, you can be next.

DR. BANCHEREAU: Thanks. Obviously, it would be wonderful if you could do that, but how can you do that? I mean, maybe one day we will -- I really don't know how to address that. It's a tough question. Ralph, why don't you take it?

DR. STEINMAN: The antibodies stem HC peptide hopefully will come on board. That may not be a simple situation though. And I think what we're doing in terms of peptide is we always have a

functional readout for dendritic cell stimulation of a peptide specific T-cell and then we saturate the system functionally. So, for example, in the flu matrix system most of the time 10 nanomolar or 100 nanomolar peptide is plenty, and yet we're treating the dendritic cells that we prime the patients with or boost the patients with ten micromolar, one micromolar peptide so we're way above saturation we think.

DR. MARTI: Go ahead --

DR. STEINMAN: We'll really get, I think, held back if we start doing all these measurements as a standard thing. It's certainly a terrific research --

DR. MARTI: Experimental.

DR. LOTZE: I just want to make a cautionary note that the peptide pulse DC in vitro is not again necessarily what you want to measure because they have their own life history after you've pulsed them. They will lose their ability to present that peptide with time, decrementally very quickly in studies shown from Franco Marenclo's group here at the NIH, within 24 hours these peptide pulse DCs have substantially decreased their ability to stimulate specific T-cells, and a recent

publication in JI from Andy Amascatto at the University of Pittsburgh showed that peptide-pulsed DCs are the Edward Scissorhands of immunology. They slice and dice the peptide very quickly in a model peptide antigen tyrosinase. You actually cleave it very quickly and the question is how long do these peptide-pulsed DCs remain as effective immunogens, and it brings up all sorts of questions about whether alternative strategies using longer peptides, authentic proteins or again as was suggested, apoptotic bodies and apoptotic cells being the preferred way to deliver antigen that we contemplate not only what happens in a heartbeat in our in vitro assays, but also what's happening over the course of DCs in vivo because I think they're degrading these peptides very quickly.

DR. MARTI: I think Dr. Urdal has a comment and then Dr. Banchereau.

DR. URDAL: I just wanted to kind of echo the remarks that Mike went on to say, there's really nothing new to that.

DR. BANCHEREAU: So suddenly one day, I believe that we're going to have before the panel a monoclonal antibody that would recognize the right peptide in the context of the right HLA Class 1 or

Class 2, but it's going to take a number of years to identify the right peptides and then to identify the right antibodies that recognize that. Meanwhile, I think functional assay is going to be the conclusion.

DR. MARTI: Moving on down, if we could still have some comments from the other end of the panel with regards to functional assays. Dr. Kufe?

DR. KUFE: Well, as I presented, we've been using MLR assays with the fusion cells as one functional assay, and we've just begun cultivating the fusion cells with autologous T-cells and then assaying the function of those stimulated T-cells to kill autologous targets at CTOs. So those are our two main functional assays that we're using right now.

With regard to antigen in the approach we're using with a fusion cell, we have a semi-quantitative way of identifying the antigens that are expressed by the fusion cell, but I think it's still problematic in terms of how much is actually presented on the cell surface as you have with peptide loading.

DR. MULÉ: One additional assay that we'll hear more about tomorrow is the MAC peptide

tetramer. Peter Lee is here and it's obviously not a functional assay per se, but allows us to very easily monitor

T-cell reactivity in bulk assays by measuring directly peptide binding T-cells as a way of giving some indication of whether or not there's skewing or biasing or the T-cell repertoire of pre versus post immunization.

DR. MARTI: Raj?

DR. PURI: I was just wondering about a question from Dr. Lotze. If that antigen is cleaved off from the MHC group in very short period of time so the functional assay which you are measuring it may not be due to the antigen or maybe soluble antigens are not presented in the MHC group or the short time exposure to the responder cell is enough to generate the functional response.

DR. LOTZE: If I understand your question, Raj, it is if you believe that the peptide is being lost with rapid kinetics from the pulsed DC, how are you eliciting an immune response? I actually think it is the peptide and the MHC. It's just that in terms of comments about how much peptide you need, it's possible that you're not only filling empties as well as displacing loosely bound

peptides in MHC, which I think is perhaps a little bit less likely that you might be also loading cytosolic stores. I think Drew Pardoll's comment was in a ridiculous way with very high antigen concentration which is what you're doing with synthetic peptide, you can actually drive it into the cell and you might be able to get some loading into Class 1, but I think it's a very inefficient pathway and we don't have any direct evidence to support that that's the major way the peptide is getting into Class 1.

DR. PURI: Is there any possibility that the peptide is recycled, is taken up again by the cell, by the passive transport and then presented again in the MHC group?

DR. LOTZE: I think Ralph presented evidence as well as Drew that DCs can generate antigen which is taken up by secondary set of DCs whether you invoke exosomes or perhaps some other mechanisms that there's cross priming which occurs at a continuous level in vivo. And so it's possible that the DCs that you're generating are also somehow leading to the direct feeding and activation of another set of DCs that are resident in the host and to whom you've transferred these cells.

MS. GOLDEN-FLEET: I have a quick question, please? I'm Meg Golden-Fleet from Wake Forest Cancer Center. Referring to a point that you made about once you have pulsed your dendritic cells and they start to decrease in their ability to stimulate T-cells, would that affect, if you wanted to generate your dendritic cells, pulse them, then cryopreserve them and use those to infuse your patients, do you think that would decrease the function if that's what you wanted to do? Or they have a very small amount of time that they would really work?

DR. LOTZE: These are almost impossible questions because, again, if you're going to use clinical endpoints as the important ones, it's impossible to know that. And we've struggled with this in our own laboratories and had prolonged conversations about whether to do just that, pulse and then freeze and then thaw and so on and my current feeling is you pulse, wash and get into the patient as quickly as possible to try and optimize the time period in which they can encounter your specific

T-cell because recognize that the DCs have got a little bit of work to do, once you do the adoptive

transfer. It's got to migrate into the secondary lymphoid organs and encounter the appropriate T-cells, and I think the least time possible from peptide pulsing to getting into the patient is probably the best, but that's intuitive, there's no data that supports that.

DR. PURI: If I may, this question reminded me that I needed to ask that question for Dr. Urdal. Dr. Urdal has suggested that CD-54 expression on DC cells could be taken as a potency test, as opposed to identity test, because you look at some sort of proliferative response to CD-54 antigen. He indicated that MLR takes seven days and it's not feasible to use that test before you administer cells to the patient. What happens -- are you planning to give dendritic cells in a repeat administration a different cycle to this patient, and if yes, then shouldn't that MLR be done in those cases to look at the functional response?

DR. URDAL: The current prototols that we're following usually involved monthly infusions or twice monthly infusions of antigen-pulsed dendritic cells, and it's a process that they basically, after the antigen-pulsed dendritic cells are created in vitro, they're delivered and infused

into the patient within six to eight hours. So any assay that would be used as a release assay that can't be done within that period of time is not one that would be very meaningful for that particular protocol that we're using. Now in all studies or most of the studies we're doing now we are, in fact, doing MLRs so we're collecting that data after the cells have actually been reinfused into the patient, but what we need are assays that reflect the properties of the cells that we think are important to their function in vivo, but that those are assays that could be accomplished within this period of time that you'd like to get them back in in their most viable and healthy state into the patient.

DR. PURI: And if I may, another question for Dr. Urdal again was that how quantitative assessment of CD-54 expression you can do by flow cytometric analysis or do you have a quantitative way of determining the antigen expression?

DR. URDAL: Well, what we see in our system at time zero the 54 population that we gate on is not present. It only appears after the 40-hour culture period that we use and so in a sense by setting those gates and seeing the level of

expression that we can measure, we're reasonably confident that we have a relatively robust way of being able to identify that population of cells in the patient samples that we've been processing up until now.

DR. MARTI: We'll have to take these last two questions very quickly. We'll start with you, sir.

DR. AVIGAN: Just a quick comment, David Avigan from Beth Israel and Dana-Farber. At the last DC meeting it was mentioned that it is really uncertain what the best maturational state for dendritic cells for clinical analysis is. And I think before we get into sort of formatting both functional or immunophenotyping because immature DCs really look very differently than mature DCs. I think that question has to really be answered, so even CD-14 which is something that we've been excluding is something that's seen in some immature populations.

DR. MARTI: Thank you. And you, sir?

PARTICIPANT: A question for Dr. Urdal. How do you avoid cross priming of your DCs by contaminating tumor cells which might originate due to the aphoretic process to collect the DCs?

DR. URDAL: At the moment, we don't avoid it. If it's occurring, we don't -- if there are contaminating tumor cells we're not really checking for them at this point so it's not something that we're actively looking at at this point.

DR. MARTI: According to the timer here I have 10 seconds to sum up the panel's discussion. There's lots of suggestions and ideas for -- I'm more optimistic of developing consensus on a phenotype than I am on functional studies. Also, it seems to me that the group would like the NCI to make some of these reagents available for study and they would like to see some support for the ELISA spot and there's a call, a general call to collect data. Let's end this session now and the third session will begin tomorrow morning at 8 o'clock, 0800 hours.

(Whereupon, at 5:02 p.m., the workshop was recessed to reconvene tomorrow, Friday, December 11, 1998 at 8:00 a.m.)