UNITED STATES OF AMERICA

DEPARTMENT OF HEALTH AND HUMAN SERVICES

PUBLIC HEALTH SERVICE

FOOD AND DRUG ADMINISTRATION

CENTER FOR BIOLOGICS EVALUATION AND RESEARCH

AND NATIONAL INSTITUTES OF HEALTH

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

WARREN GRANT MAGNUSON CLINICAL CENTER NIH

GRANULOCYTES FOR TRANSFUSION: RESEARCH AND CLINICAL EXPERIENCE

WORKSHOP

Friday,
September 11, 1998

The workshop took place in the Jack Masur Auditorium, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892, at 8:00 a.m., Liana Harvath, Ph.D., Chair, presiding.

PRESENT:

KATHRYN C. ZOON, Ph.D., Director, CBER
JOHN GALLIN, M.D., Director, WGMCC
LIANA HARVATH, Ph.D., Chair
DAVID STRONCEK, M.D., Moderator
DOUGLAS ADKINS, M.D., Speaker
DANIEL AMBRUSO, M.D., Speaker
DAVID DALE, M.D., Speaker
JOSE-LUIS DIAZ, Ph.D., Speaker
THOMAS LANE, M.D., Speaker
SUSAN LEITMAN, M.D., Speaker
CONRAD LILES, M.D., Ph.D., Speaker
THOMAS PRICE, M.D., Speaker

ALSO PRESENT:

AGENDA

Welcome/Introduction
Kathryn C.Zoon, Ph.D., Director CBER/FDA 3
John Gallin, M.D., Director, WGMCC/NIAID 5

SESSION I
Moderator Liana Harvath, Ph.D. 7

Historical Perspective & Clinical Trial Considerations
David Dale, M.D. 9

Cytokine Administration to Normal Granulocyte Donors
Thomas Price, M.D. 47

Functional Properties of Granulocytes from Normal Donors after G-CSF Administration
Daniel Ambruso, M.D. 71

Cytokine Mobilized Granulocytes: Assessment of Efficacy
Susan Leitman, M.D. 91

Panel Discussion 120

SESSION II
Moderator David Stroncek, M.D. 154

Product Evaluation
David Adkins, M.D. 154

Storage Considerations
Thomas Lane, M.D. 175

In vitro Assays Predictive of Product Function
Conrad Liles, M.D., Ph.D. 197

Panel Discussion 216

Presentation of Submitted Abstracts
David Stroncek, M.D. 229
Dr. Diaz 242

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

(8:08 a.m.)

CHAIRPERSON HARVATH: Good morning. I think we will get started in the spirit of trying to keep on time. On behalf of the Steering Committee for this workshop, it is a pleasure to introduce two very distinguished individuals, Director for the Center of Biologics Evaluation and Research, Dr. Kathryn Zoon, who will give welcoming remarks, and Dr. John Gallin, who in this audience really needs no introduction since his career in the granulocyte field has made enormous contributions to this field. Dr. Gallin will be speaking on behalf as the Director of the Clinical Center as well as making remarks on behalf of NIAID. Dr. Zoon?

DR. ZOON: Good morning. It is a pleasure to be here and to open this important workshop with Dr. Gallin. John and I have known each other many years, in our interferon days and working on various activities of interferon gamma and granulocytes. I think there is lots of work to still be done and I am very anxious to hear the science today and understand where we are with the technology and where we need to go tomorrow.

It is a pleasure to welcome you on behalf of the Center for Biologics Evaluation and

Research, which is one of the FDA centers that has oversight of blood and blood products. This workshop today, I believe, will really help us focus on some very important issues. The findings that the administration of cytokine, such as granulocyte colony stimulating factor and granulocyte macrophage colony stimulating factors to normal volunteers results in the peripheral mobilization of high concentrations of granulocytes have renewed the interest in the collection of granulocytes for transfusion. I think all of us have become very familiar in the literature with both the effects of G and GM-CSF on this. And while there was a lot of interest in granulocytes for transfusions -- this peaked back in the 1970's -- there is now a renewed interest because of our new tools. We are very interested in exploring both the efficacy parameters as well as the safety parameters associated with this.

We are very interested, and of course there are many others interested, in the scientific and clinical experience with cytokine mobilized granulocyte transfusion products and the effects of the cytokine administration on normal donors. This is clearly important with regard to the safety of the patients and the donors, but also important in

the quality of the products, in this case the granulocytes looking at their functional capabilities.

Our colleagues at the NIH are here today to hear what areas in this field need to be further explored and supported in the research area, and we really are very appreciative of doing this workshop today in a collaborative effort, because many of the fruits of the science and the research that do arise come to FDA for our review and evaluation, and it is very important that the Agency be very active in the science and understanding the science so that we can do the very best job at facilitating the review and access of these important products.

This is the second workshop this week. We had one yesterday on hematopoietic stem progenitor cells and this one today on granulocytes for transfusion, and we really appreciate the attendance today. We feel this is a very important area. We feel very strongly that we need the best scientific data in which to move forward, and I wish you a very good meeting and look forward to the fruits of this workshop. Thank you very much.

DR. GALLIN: Well, normally I don't know that I would get up and be willing to share in two introductions to one meeting, but for this meeting I

couldn't resist. And the reason is because of my own longstanding personal affection for the granulocytes, which are by my way of thinking the most beautiful cells in the body, and also because of the importance of granulocyte transfusions. So on behalf of both the Warren Grant Magnuson Clinical Center and all the staff who work here as well as the National Institute of Allergy and Infectious diseases, both of which are places that I work, we welcome you here.

Now my personal interest really goes back to the use of granulocyte transfusion in patients with granulocyte defects, particularly patients with chronic granulomatous disease and a few patients that we have seen in this building with neutrophil specific granule deficiency. And over the last 25 years, we have been convinced, truly on anecdotal evidence, that there are some patients in whom granulocyte transfusions made a difference in helping them get over life-threatening infections. But, of course, there is no proof that they work, and that has always been an issue. And we have also been worried that maybe we were actually doing something bad or potentially bad. Perhaps we always worried that maybe we would be precipitating ARDS in patients with chronic granulomatous disease or that

we would be alloimmunizing the patients to an extent that future bone marrow transplants or gene therapy might be compromised.

Nonetheless, we continued to use them on occasion. So I personally really look forward to the results of your deliberations today with the hope that you will come to some conclusions in terms of not only the correct approaches for mobilizing these cells and harvesting them and storing them, but also for designing some clinical trials that will answer some of the definitive questions. So have a great symposium.

CHAIRPERSON HARVATH: This is a great pleasure for me to be involved in helping to organize such a workshop, because my own area of research for the past 25 years also involves those beautiful cells of the body, the granulocytes.

In looking through our sort of cryptic files of workshops at the FDA, I came upon some transcripts of a workshop held in October of 1980 in Natcher Auditorium, and it was called the Conference on Leukopheresis Donor Safety. And that was actually the last conference that FDA was involved in where there was a discussion of the collection of granulocytes and leukocyte products, and the discussion at that time focused on donor safety

issues when donors are given hetastarch or when they are given steroids for mobilization.

There were a number of participants in that workshop who were actually serving on the Steering Committee. So what I would like to do is to in the next slide -- what I had is a list of people. There should be two slides in that carousel. The second slide -- there we go, thank you -- is to acknowledge and thank the members of the Steering Committee. All of these people have distinguished themselves in the granulocyte field, and I would like to go through this list. Dr. Daniel Ambruso from the University of Colorado and Bonfils Blood Center will be speaking to us; Dr. David Dale from the University of Washington; Dr. John Gallin; Dr. Jeffrey McCullough, from the University of Minnesota, who unfortunately can't be here but who made major suggestions for the organization of this workshop; Dr. George Nemo from the Heart, Lung and Blood Institute; Dr. Daniel Rotrosen, who worked in this building with Dr. Gallin and Dr. Malik in neutrophil research; Dr. Ron Strauss from the University of Iowa, who is one of the pioneers in this field, who unfortunately can't be here because he is giving a talk on granulocyte transfusions at the Pediatric Oncology Meetings

today in Chicago; Dr. David Stroncek, who now works here at the NIH Clinical Center, and you will hear some of his work this afternoon; and finally, our colleague, Joseph Wilczek, who has served as the program coordinator in taking care of the laborious details that go into putting a conference like this together. It is a great pleasure for me to have the opportunity to introduce the speakers in the morning session. Dr. David Dale began his interest in granulocytes almost 30 years ago in this very building, where he was working as a clinical researcher, and he is now a professor of medicine and has had a very distinguished career in education and research in this field. Dr. Dale is going to talk about the historical perspective and clinical trial considerations for granulocytes for transfusion.

DR. DALE: Well, thank you, Liana. It is nice to be here. I would say nice to be here again. I think I last spoke about this topic in this room 25 years ago, so it does bring back a lot of memories.

If I can have the first slide, which is just a title slide. I am going to talk this morning about really three things. I am going to talk about history. That is always a dangerous thing to do. I

will mention a lot of names. I may not mention everyone because of the time, but there have been many people involved over the years in studying this topic, many who have done other things in their lives and you will recognize them. I am going to talk a bit about the effects of G-CSF in particular on neutrophil formation and function, again a part of the background for our overall discussions today. And then finally talk about recent history, and that is the data that has led up, I think, in many ways. And then finally to mention a little bit about clinical trial considerations.

Our focus today is on the neutrophil, which my predecessors here this morning have described as beautiful, and they really are interesting cells. Most oncologists see them on a laboratory slip as a number. Hematologists may see them on a blood smear. But they are truly interesting and beautiful cells. Perhaps made more interesting and beautiful if you see them in a diagram like this, which shows some of the features of a neutrophil. I won't dwell upon this today, but suffice it to say that there are many features of these cells that are regulated very tightly, and the cells that circulate in the circulation are not

always the same. Infection, the administration of hematopoietic growth factors, diseases, many factors can change the characteristics of these cells. The key features though are the surface of the cell, where the cell has receptors which allow it to interact with its environment, the granules of the cells, which are critical for the cell function, and then, of course, the nucleus, which allows us so easily to recognize a neutrophil in a blood smear.

In terms of the formation of neutrophils, as a background statement I think it is very important to think about the kinetics. Because the dynamics of how the body produces and how neutrophils are distributed in the blood and their ultimate fate have so much to do with the development of this field, both in the past and in the future. Neutrophils are formed from hematopoietic stem cells and the steps of proliferation and differentiation and then maturation are very unique for these cells. The most unique feature compared to other blood cells is the storage in the marrow of a substantial portion of the body's total supply of the cells. How much is it? Well, it is probably 10 times the circulating supply or perhaps more depending upon where you draw the line. But suffice it to say it is a very large

reserve, a reserve that is not there for monocytes or eosinophils or red cells or other of the hematopoietic cells. And it is really this reserve supply of neutrophils in the marrow that are critical for the development of the whole concept of collecting neutrophils from normal donors for transfusion. Neutrophils, as you will recall well, have a short blood lifespan, and in fact almost all of their function are in the tissues. And although clinically over many years we have related susceptibility to infection to the number in the blood, it is actually the total body supply and the ability to deliver these cells to the tissues which is critical for the outcome in terms of the problem we are talking about.

And then finally as background, I will mention the process for the killing of organisms, represented by this pink cigar here, by a neutrophil is a complex process that we have unraveled in research supported here and in much done here as well as elsewhere around the world, to clarify the various processes involved in the dumping of myeloperoxidase into the phagocytic vacuole and the involvement of oxygen and oxygen derivatives in the actual killing of these organisms. And it is this event, actually the ability to kill organisms, that

is why we are all healthy enough to be here today. So it is vitally important not just for sick people but for people who regard themselves as healthy.

Now critical finally for the understanding of this topic is the short lifespan or the rapid turnover of neutrophils. This is illustrated here just by comparison with other blood cells. Red cells having a lifespan of roughly three months or a turnover rate of 1 percent per day, platelets roughly a tenth of that in terms of their lifespan, and ten times as rapid turnover, but neutrophils are among the most rapidly turning over cells in the body. In many audiences I have described it as just think of the fact that in your blood today, you have a whole fresh supply of neutrophils from what you had yesterday. You have replaced all of those cells. And if you go just a few days without a new supply, of course you are in trouble, as we all know.

Now in terms of thinking of history, this is actually an old slide, I think one that I showed here in a slight variation 25 years ago. The problem of neutropenia has been one that has been recognized for almost the whole 20th Century. The problem was actually recognized soon after the turn of the century, with neutrophils being counted in

blood smears over the few years before but really not very long. But the treatment of neutropenia has languished. This is basically our 1970's approach, that is, the use of antibiotics, and we still depend upon them to deal with the problem of neutropenia. We had then in the 1970's a variety of agents to try to increase neutrophil production, I would say all very, very weak. There were efforts to try to treat neutropenia with other factors such as removing the spleen in chronic states, but of course this was not applicable to acute states as occurs after chemotherapy. And finally then, as now, a key concept in the treatment of neutropenia was an alternate supply, that is, to transfuse the cells.

In terms of the history of this idea, that is, the history of transfusing neutrophils to treat neutropenia, most reviewers of the topic would point to a study done in 1934, the height of the Depression, a study involving injection of buffy coat cells into patients, intramuscular injections, which were undoubtedly very painful and basically had no effect on the patients. But it was a part of a desperate approach in the pre-antibiotic era to doing something about the problem of neutropenia.

Actually, the first important investigation in this area was conducted here by Dr.

George Brecher and associates, working with Gene Cronkite, who was then at the Naval Medical Center, and that study, I think, was a landmark in terms of the building of the basic physiological concepts underlying this field. What Dr. Brecher did -- I don't know, somebody in the room may know him -- I remember him from when I first came here, a really great man. What he did were studies where donors, that is, dogs which had been made aplastic with radiation -- the donors were injected with turpentine to try to increase their counts and then the recipients were irradiated to aplasia, and then cells were separated and it was shown that cells could be accumulated at the site of inflammation. In Brecher's original studies, he showed that some cells could get there and they could circulate and that some effects could be seen.

What happened historically from that point was really very gradual. But a key event again here in the early 1960's was the development of the concept of transfusing cells from CML donors. Studies that were, I would say, led by Jay Freireich but involved a number of people here and subsequently elsewhere, showed that you could take CML cells, donors were patients who were untreated and recipients were patients with leukemia usually,

and you could get the CML cells to circulate in those persons and the more mature of the CML cells would actually migrate to a site of inflammation. More dramatically in those early studies it was shown that using a very simple index that the patients became afebrile. Dr. Freireich often talked about the fact that patients treated with CML cells showed clinical evidence of improvement very rapidly. Those studies were greeted with great enthusiasm. I would say the enthusiasm was tempered in time as treatments for CML improved, and also it was recognized that you could have the CML cells engraft, you could transfer infection with CML cells, and that there were a variety of complications that were associated with this approach to therapy.

Actually, the next period of development centered on the development of the cell separator, work that was supported and performed here by a series of investigators. I would say Dr. Seymour Perry, who many of you may remember worked here for many years studying granulocyte and leukocyte kinetics, was the real father of the NIH efforts in this regard. But there were a series of investigations performed here by Dean Buchner, Bob Epstein, Bob Graul, and then myself over a period of

years which really tried to lay a physiological framework for the advancement of neutrophil transfusion. This is a picture from one of the first papers about this topic of the centrifuge for collecting cells. And we all know that have been involved in blood banking how centrally important the development of this centrifuge concept for separating cells on the basis of their density has been. So it was great research done by the IBM in collaboration with the NIH, and it did lead to the capacity to procure large amounts of cells.

This is a very brief slide about Dean Buchner's work, studies which were originally done in dogs, and showed that you could collect of the order of 24 billion white cells if you kept a dog on this centrifuge long enough, and that you could then actually transfuse these cells and see them circulate, sort of reproducing the work that George Brecher had done, but showing it with larger numbers of cells in the same species, but showing that you could, in fact, get very good increments if you used enough cells. And importantly, they showed in this very early study that cells collected with a centrifuge would circulate. And subsequent to this work, Bob Epstein, who worked in Seattle with Reg Clift and Don Thomas went on to show that you could

use this same model and then in the irradiated dog injected with E.coli to develop bacteremia, you could in fact show an effect on an experimental infection in terms of the clearance of the infection.

Now when I joined and began working with Bob Graul, he was then actually in that era just beginning clinical trials here of the use of neutrophil transfusions for patients with sepsis. These were pioneering studies, studies that were done not in a rigorous controlled trial but studies published in the New England Journal, which clearly suggested that this was a promising area for application. The original studies were done primarily but not exclusively with centrifuge collected cells. The studies that I was involved in here with Bob and Herb Reynolds and a number of other investigators involved dogs, again irradiated to produce neutropenia, and then injecting those dogs with Pseudomonas aeruginosa intratracheally to cause a localized pneumonia, and then treating the dogs in a randomized controlled rigidly monitored study where some dogs were supported with platelets only and the others were supported with platelets with granulocytes.

Just to make it a little more interesting and colorful, I can comment very briefly upon things I remember about the trial. As I was walking around here yesterday, I was remembering I used to keep an old bicycle outside the cafeteria downstairs to ride back and forth to the animal quarters to administer all the treatments to these dogs. And a key reason for our success in this trial is that the NIH then had a farm in Poolesville. I think it is all developed now, but we had a farm out there with great big foxhounds who were the donors, and in our clinical trial or our preclinical trial, we used very small beagles as the recipients. So that allowed us the advantage of despite the number of cells we could collect, we could see and measure and do a lot about neutrophil increments.

This is the picture of the lung of a dog injected unilaterally with pseudomonas and developing a characteristic hemorrhagic pneumonia in an animal with a very low neutrophil count. The key observation we reported in these studies in the Journal of Clinical Investigation in 1974 is in fact that if you looked at controls versus transfused animals, you could clear the Pseudomonas of the specific type we had injected from the lung by a

series of transfusions. And we showed in our randomized trial that you could improve survival. A number of other things came from the study, but it was the place that I personally became convinced if you had enough cells that were functionally intact that you could use this approach to treating severe infections.

We also studied at this period the interaction of antibiotics and neutrophils and derived certain conclusions about that, and I think that is another important consideration that will come up when any clinical trial is now considered, that is, which antibiotics are best and how to approach the antibiotic neutrophil interaction.

In terms of what happened then is a nice illustration, I think, of the circuitous path of clinical research. This is a picture of a 1970's filter that we used and was used widely then to collect neutrophils by filtration leukopheresis. Many of you will remember this. It basically depends upon the property of neutrophils to stick to anything almost, and that is in fact the way that they are selectively recruited to a site of inflammation, and this basic stickiness of the cells was how they could be collected in much larger numbers. Many of the studies performed in the

1970's involved the use of this filtration system as a way of trying to increase cell numbers.

And what happened is that in the development, it was learned that you could improve collections if you used starch to accelerate red cell sedimentation and if you used steroids to raise the counts in the blood. But what really helped in terms of the numbers was the use of a filter to get lots of cells. This is data from that era showing a comparison of how many cells you could obtain at best with a centrifuge and how many more you could collect by filtration leukopheresis. These are probably conservative differences. That is to say if it were three to five times as many that that would be the expected. The problems that occurred were that although the efficiency of collection was more, the cells were damaged in the process of their collection. And in fact probably in the process of activating cells, we would say in modern terms the release of cytokines from neutrophils, you often saw febrile transfusion reactions in response to the administration of filter adherence collected cells. Nevertheless, until around 1980, this technique was widely used.

There were a number of studies in this era that sort of took on, if you will, the

filtration leukopheresis cells and looked at what was wrong with them. This is a paper by Tom Price, who is speaking next, about this, looking at the difference in the disappearance rates of various types of neutrophils, cells from phlebotomy, cells collected by centrifugation, intermittent flow centrifugation, and then cells collected by filtration leukopheresis. And basically the cells were -- it was found that the cells were damaged enough in the collection process that they wouldn't circulate. And although there was suggestive evidence that they might be useful for therapy, the evidence was never very strong. And particularly because of the transfusion reactions, this process of collecting cells by filtration leukopheresis fell out of vogue.

Now there have been many summaries of the studies that were done in the 1970's and early 1980's looking at the benefit and the use of granulocytes based on various trials. This is a slide borrowed from the summary work by Ron Strauss outlining what he would consider the best of these trials, dating from Bob Graul's trial that I mention here as the first of these, up until a trial performed at UCLA in 1982. So it spans a 10-year period. Those of you interested in clinical trials

will say aren't these pretty small numbers, and they really are. In fact, the number of patients in the treated groups are sufficiently small that part of the problem with these trials was just basically their size. Another problem is that cells in these trials were collected by various techniques, and in general the trials that showed the best results involved the transfusion of the largest numbers of cells.

Another of the problems in these trials is that the patients weren't always the same. That is reflected probably most easily here if you look at the percent survival of the control groups. Because if the comparison group did well, it was very difficult to imagine that you would show a benefit of the treatment. So, for instance, in a study like the last study with a 72 percent survival rate in the control groups, the fact that the transfused group did more poorly, these numbers are probably not different, but this is so high it is hard to imagine that this trial would have shown a benefit. Suffice it to say the clinical trials were not sufficiently convincing that although there were people who spoke enthusiastically about this topic for a number of years, clinicians in general dropped this idea because of the results of these randomized

trials and the difficulty in seeing the benefit to a patient of a single transfusion.

If you look in more detail at these trials though by specific types of infection, you can see that for certain kinds of infections it appears that patients really did do better. And I would say that Ron Strauss, who wrote this report, were he here would say that he believes that what these trials showed is a benefit, but that the trials were not sufficiently convincing to convince everyone of that.

Probably the most positive and most recent trial of note is the trial that was performed by Mitch Cairo and associates, a trial that involved an interesting comparison. These were in children, so you had the advantage of small recipient/big donors, but you also had a comparison group, that is, the comparison of neutrophil transfusion versus a control which involved gamma globulin injections in neonates. What this trial showed is that there was a very significant benefit in neonates, but critics of the trial have said that the two groups in the study were not really comparable and that the methods of randomization lacked the rigor to make this study really a definitive study for the treatment of neonates with sepsis, and in fact it is

for this reason probably that this trial was never really accepted as convincing evidence or the practice of neutrophil transfusion in neonates, despite these very striking results, has never been widely introduced.

Now paralleling these efforts by many people to develop neutrophil transfusion was another development that is very important for the reason that we are here today. And that is the development of our understanding of the regulation of granulocyte production and the use of the colony stimulating factors in patients and in normal subjects to try to raise the neutrophil count. And because it is so basic to our discussions, I thought I would review this background information with you as well as a part of my history talk.

Many of you will recall that in the mid-1960's, it was learned that you could take bone marrow cells in a petri dish and with a tissue culture media and some source for the stimulating factor, the cells would grow and form colonies. Again reflecting back, it was during my years here that this technique came along, and it was a very exciting development with Paul Carbone and Clarence Brown. We did the original colony assays here at the NIH showing that you could grow cells because

this led to so many important developments in the whole field of hematology, oncology, and transfusion medicine.

Suffice it to say the field has come a long way. And I would like to make just a couple of important points related to this slide about the various growth factors involved in regulating leukocyte production. The key concept is that early cells have lots of surface receptors affecting the formation of cells, but the late stage of development, or more specifically, the regulation of the number of circulating neutrophils is governed by a single factor, G-CSF. That is to say that an animal made deficient in G-CSF does not have a normal circulating count. And although they make neutrophil precursors, they don't mount a normal neutrophil response usually or in response to infection. So the G-CSF, one of the reasons that we are talking about it today is that it is the natural regulator of the level of circulating neutrophils in the same way that erythropoietin is the natural regulator of the circulating level of red cells and thrombopoietin is the natural regulator of the level of circulating platelets.

Now G-CSF as a drug was introduced in the late 1980's, and many of you know a great deal

about it. The gene was originally isolated from a bladder cell line, a cell line that was from a patient with neutrophilia. In fact, many malignant cells overproduce G-CSF and served as the original source for the material that was used in developing the basic structure of this protein and understanding its genetic regulation.

The pharmacological effects of injecting G-CSF are now well characterized and most of you know then. That is, if you inject this drug, you can quickly achieve levels that are far higher than you normally achieve with infection or stress or a variety of other natural stimuli. The drug is prepared and is easily administered and in fact has relatively few side effects. This will come out further as the conference proceeds.

In terms of how the colony stimulating factors work, just a few key points. One is we heard a lot yesterday about the use of G-CSF, and you could say that for GM-CSF too. That is, they are agents which mobilize the earliest hematopoietic cells from the marrow to the blood. The details of exactly how that works are still not know. But it is a dramatic effect, an effect that was totally unexpected when these agents were originally studied.

G-CSF is particularly powerful also to stimulate the flow of cells down the pathway or as I usually describe it, to squeeze down the production time for neutrophils from early cells to mature cells in the marrow. So like an accordion, you squeeze down this time dimension and you get more cells and you get them quicker. And finally, because you have more cells -- well, G-CSF also releases the mature cells from the marrow to the blood and then finally because you have more cells in the circulation, it allows for the possibility of a larger inflammatory response. So you can see that this natural stimulus, that is, it arises in infection, or as the drug might be used to stimulate neutrophil production has a multiplicity of effects. And you can imagine that in the development of this agent that there have been many potential clinical applications. We are talking today just about one of them.

We began in Seattle to try to investigate and to build this picture further, now about 8 years ago, about 1990. Our original studies were a trial that we did to try to compare the effects of G-CSF in young and elderly subjects. Our original idea was to try to study aging. That is, we wondered if there was an impairment of the

proliferative capacity of cells as people get older. So we compared healthy young people and healthy elderly people and we had a regimen which involved G-CSF daily for two weeks and then a whole variety of measurements. I am not going to dwell upon many of these, but just to show you some highlights from studies that we have done then over the last eight years.

This was the original curve showing that normal people have a very stable neutrophil count with no injection. If you give a small dose of this drug, you get a small effect. If you give a larger dose -- this is 30 mcg total dose per day and this is 300 mcg total dose per day injected once in the morning subcutaneously measuring morning counts, and what you see are these characteristic patterns. And if we had gone up higher in the dose, there probably is a plateau, but no one has ever really measured how high that plateau may be in terms of driving neutrophil production with this drug. But you can see with a dose of 300 mcg daily, you can get to a plateau count of roughly 25,000 in healthy people fairly quickly. And in fact it is this rapid increase which distinguishes the effects of G-CSF from GM-CSF when administered to normal subjects.

GM-CSF causes a much more gradual rise in the count over a more protracted period.

If you look at the cells that are produced in this kind of a setting, show on the left-hand side are normal neutrophils and the same person's blood looked at 5 days later after daily administration of G-CSF. It is a gradual transition. But what you see is the production of bigger, bluer cells with a somewhat less mature nucleus. And if you look in greater detail, you can see a number of other interesting features of the cells. These are sectioned electron micrographs which show normal neutrophils and cells from a person treated for five days with G-CSF. And as you can easily see, the cells are bigger. If you look more carefully at the cells, you can see the surface of the cells are smoother. You can see in these cells bits of endoplasmic reticulum or what would be referred to in a laboratory as delivery bodies. You can see differences in a variety of things, including probably the average size of the granules. Suffice it to say the stimulus changes many aspects of the cell morphology, but in general produces cells that are younger looking.

If you look by scanning electron microscopy what you see are if this is a normal

neutrophil with its rugged surface -- this is a neutrophil from someone treated for 5 days with G-CSF. There is more redundancy to the membrane of the cell, and you can see lots of these cells that look like this with scanning electron microscopy. And it is a reflection of the fact that as neutrophils mature, like as people mature in general, they shrink and the membrane shrinks around them. So these changes are not totally unpredictable in terms of general cell biology, but they are rather dramatic to look at. I have often described these cells as looking like someone running down the hall with their white coat flapping behind them. And there are probably many features of how the cell functions that are slightly different for these cells versus these cells, but in general the cells have the same basic function.

Tom Price and I did studies in this era of investigation looking at how much does G-CSF stimulate the flow of cells from the marrow to the blood. And this is kind of a classic study, something I learned to do here from Seymour Perry. Studies which show if you injected tritiated thymidine and look at the yellow curve here, this is the normal emergence time for a neutrophil from the marrow. That is, you label with tritiated thymidine

the cells at the last stage of cell division, and then watch for those cells to appear in the blood. There is normally a lag time of about six days. This has been known since the 1950's. If you inject G-CSF, what you can show with our studies is that you shift this curve to the left. The 30 mcg curve shifted it this much and the 300 mcg curve shifted it this much. That is, you reduce the post mitotic transit time for the neutrophil from roughly 6 days to 3 days or reduce it by 50 percent. That is a big change, and so much of what you see reflects this pushing of the cells down the pathway and getting them into the blood sooner, younger, and looking as I just showed you.

If you look at a schematic of what happens when you give G-CSF to a normal person for a period of time, you go from a schematic that looks like this with each of these bars representing a cell between divisions, and the number of divisions reflected by the number of forks along the road. You can see that if you give G-CSF, either as a drug or if people produce it in response to infection, you amplify the number of cells produced, and you do it in a shorter period of time by reducing primarily the G-zero phase of cell development.

If you look at the function of these cells -- we have done a number of studies and this is terribly relevant to the development of this field. If you look at neutrophil function after administration of G-CSF, what you see depends upon when you look. Because you are looking at a dynamic circumstance. Now this is a graph from work we published a few years ago, work with Bob Allen. Other people have done this general line of work. But if you look at the three groups of subjects from this trial we did -- control, 30, and 300 mcg treatment -- and if you use a low stimulus like PMA in a low concentration, what you observe is that cells from the blood of a person treated with G-CSF are primed but they are not actually stimulated or activated by the treatment. And that is to say if you take the cells from the blood and you expose them to a low dose or a low amount of this stimulus, you really see no effect of treatment. However, if you use a high dose of PMA or some other agonist, what you can show is that there is a time-dependent change in the response of the cells to the stimulus. Now these colored bars at the bottom are just a reproduction of the data I showed you a moment ago about emergence time. The purple is the shortest emergence time, which refers to the highest peak

here in terms of a change in the PMA response of the cells. And that is to say if you give a higher dose of G-CSF, you get cells produced in the blood which are, again, not activated, but they are primed to a greater degree to make a bigger response to an agonist like PMA.

Now why does this happen? I think a basic underlying concept is that if you administer a powerful cytokine like G-CSF, you actually alter many aspects of the cells. And we believe this occurs because of effects on coordinated gene expression. Not only are you inducing cell division, but you are actually inducing the enzymes that are packaged in the granules to be different than they would be normally. And we believe that this reflects, in fact, a plasticity in the production of neutrophils that occurs with infections and that is simulated by growth factor administration, so that the effects of treatment, like infection, are actually to produce cells that are more effective than normal cells would be in adaptation of the host, as we have learned over many years occurs in tuberculosis and in other kinds of infectious diseases.

Now if you look at the cell surface. I showed you pictures -- if you look at the cell

surface and look at the various properties of cells, a number of investigators have shown this. What you see, again, in terms of surface properties of neutrophils depends on when you look. If you look at one day of G-CSF treatment, actually the circumstance for most treatment and then transfusion studies, you find relatively little change. If you look at longer periods of time, though, you can see effects on various markers for neutrophil adherence and for function. And shown down here, for instance, in this corner is the substantial enhancement of the expression of CD14, a binding moiety for endotoxin that is induced on neutrophils by G-CSF treatment. Probably greater than any of these is the effect on the expression of the high affinity receptor for IgG on neutrophils, which is greatly induced by G-CSF treatment. And it is conceptually important in terms of the internalization of bacteria by neutrophils and their killing of the organisms. But the full benefit of that effect is not really known.

In terms of some effects of these changes, though, there is an interesting experiment that my colleague Conrad Liles did and published just last year. This is looking at the killing of fungal organisms, a focus of interest in neutrophil

transfusion therapy, and looking at the effects of G-CSF treatment of a normal person or potentially a normal donor and looking at the capacity of the cells to kill this class of organisms. And what this shows is the purple bars being the controls and neutrophil killing then for three classes of organisms -- Candida, Aspergillus, and Rhizopus. And what this shows is if you look at neutrophils from a normal person after five days of G-CSF, there is really no effect on the killing of Candida, which are relatively easily killed by a neutrophil. However, for Aspergillus and Rhizopus, you can show in this kind of a model the induction of an enhanced capacity to kill these organisms. These experiments were done with spores. We are currently doing experiments in Seattle now looking at the hyphae forms of these organisms. But suffice it to say that there is considerable evidence to say that you can use cytokines not only to enhance the number of cells the body produces but also the functional capacities for critical functions like this of these cells.

This is sort of a summary of what I have told you. G-CSF in this setting and what is relevant is it increases production by accelerating release of cells leading to the shift of band

neutrophils into the blood and other slightly immature cells. The cells that are released are primed for an enhanced metabolic response. If you use the right organisms, you can show that you have enhanced microbicidal activity, and actually there are a number of other changes that occur, most of which are changes which would enhance the body's response in an infection.

Now one of the most interesting effects of cytokines on neutrophil production that is important in the development of our concepts today is the effect on cell viability. This is a panel from work that Conrad Liles did a few years ago actually looking at just taking a test tube of blood, if you will, or isolated neutrophils and looking at how long those cells survive in vitro. What this shows is normally the blue line neutrophils poop out, right? You know that. If you leave a tube of blood in your pocket and forget to do a count today and test it tomorrow, the count is lower. Neutrophils die by the process of apoposis, their natural process of death, and you can show this in the laboratory very nicely that they fall off over time. This has been a central issue in the conceptualization of how you would ever supply neutrophils for transfusion therapy because they

don't last very long. Well, what has been shown by a number of investigators now is addition of G-CSF and GM-CSF or interferon gamma, these are all agents which tend to prolong the in vitro survival of neutrophils. And as I will show you in a moment, they also promote the in vivo survival of these cells.

Now we took these ideas first to the clinic in about 1993 in work that was performed at the Puget Sound Blood Center in Seattle and at the Hutchinson Cancer Center, and actually there were a number of other groups around the world who were interested at the same time -- a group of investigators here and in several other centers particularly, including the M.D. Anderson Hospital in House. The basic idea that we investigated in Seattle was the concept of providing neutrophil support for a person after bone marrow transplantation to keep their counts from going low. And because we were concerned about alloimmunization and other problems, we used the actual marrow donor, an unfortunate circumstance where we had some twins and syngeneic individuals, so that we could try to optimize neutrophil support through a period of neutropenia using cells collected from G-CSF stimulated donors.

This is a summary of the basic plan. We had seven people. We used G-CSF at 5 mcg per kilogram per day. The people donated an average of, as you can see, just over 7 times. We collected cells by centrifugation and used starch to accelerate red cell sedimentation. And because it was being done repeatedly, these volunteers became patients in a sense as they had a subclavian catheter implanted. The controls were historical controls of other people not given G-CSF. These were the rather dramatic results of this trial, which was published in Blood in 1993. Bill Bensinger is the senior author.

What the trial showed is compared to no G-CSF, that the number of cells that were collected were roughly tenfold higher. And more importantly, the increments in the blood of the recipients were almost tenfold higher, with counts measured 24 hours after the transfusion. Now if you are familiar with this field, you know that for many years, you could transfuse lots of cells but you couldn't count them. In fact, it was interesting to review some old papers. If you look at determining hematopoietic recovery after transplantation even though you are giving granulocytes, it wasn't difficult in the old days because you could transfuse the cells. There

were never any in the circulation, so you could still see when the marrow recovered.

Actually in this trial and in I would say other work since then, what you find is you get enough of an increment with transfusing cells from a G-CSF stimulated donor that it makes it hard to recognize when recovery occurs. As you can see here, we found in this trial transfusing roughly 40 billion neutrophils a day that we got a median increment at 24 hours approximately of 570, and a mean increment of nearly 1,000.

Now we weren't satisfied. And actually the following summer, using a medical student for a graduate honors project, we conducted a randomized trial of giving G-CSF with and with dexamethasone to see if we could use these two agents together to get the counts even higher. Now being interested in this field for a long time, I was skeptical that it would make any difference, but I thought it was worth a try. This is the schedule we used, chosen somewhat arbitrarily. We used the dose of G-CSF 300 mcg that we had used before or twice this amount with and without 8 mg of dexamethasone. This was administered subcutaneously and this orally simultaneously, and all we did was to do blood counts over the next 24 hours. But what you can see

is if you look at the 12-hour points, that the addition of dexamethasone to G-CSF substantially increased the levels of the counts. Or as you can see also quite easily, we went in normal people from a count of 4,000 to 40,000 in 12 hours and they barely knew it. Most of what they noticed was the effects of the dexamethasone.

So we were very impressed at how much we could raise the counts. It is still not known why this occurs. My bet -- but there is on data to prove it -- is that the steroids actually effect the capacity of the cells to be mobilized with the G-CSF. Probably some effect on receptor or post-receptor mechanisms of the cells. Suffice it to say it is a big effect and again it has potentially a large effect in planning or conducting a clinical trial.

What we did was to go on and conduct some studies using this combination of drugs, collecting cells, and making measurements. This work is sort of barely history, but it is published in August of this year in The Journal of Transfusion. Here are a picture of the cells collected in this way, nice-looking but young-looking cells. This is just giving G-CSF and

dexamethasone and collecting cells 12 hours later. And Dr. Price in a moment will talk more about this.

If you look at the general effects on these cells, and Dr. Liles this afternoon will describe this in greater detail, but you can collect 75 to 100 billions cells now with this technique, which have normal functions, slight changes in their immunophenotype, and again, as I mentioned, an effect on the survival of the cells. What we have shown with isotope labeling studies is in fact that going from a normal half-life of around 8 to 10 hours for neutrophils, the neutrophils collected in this fashion have a blood half-life of roughly 20 hours. So they have a long survival, as I showed you in vitro, and the calculated production rates or turnover rates would be, of course, very large for large increases in the cells with a long survival in the blood.

Now just as a transition to what I am going to say about conducting a clinical trial, it is very important to know that there is enough data now to say what happens if you transfuse cells like this into patients. We have done some studies in Seattle transfusing cells from people treated with G-CSF and dexamethasone to patients who are marrow transplant patients with serious bacterial and

fungal infections. This is just to highlight this information, but what it shows is if you transfuse a person with almost no neutrophils, you can bring their count to near normal with a transfusion and you can then go up another notch if you give a second transfusion the next day. That is, you can normalize the neutrophil level of a person with no neutrophils, something that heretofore was never possible.

It is based upon that I feel and I think several people here feel like it is time for a real reconsideration of this idea or a consideration of a clinical trial to evaluate neutrophil transfusion again. 20 years have gone by since this was really undertaken, and there have been a lot of changes in many aspects of medicine which makes this justified. The best choice, although challenging to do, is a randomized control trial using therapeutic transfusion rather than prophylactic transfusion. The biggest problem with this is alloimmunization if you give cells early. So late after transplantation when you really need them, the patient might have a smaller response. And also logistically this is a huge undertaking.

In general, the focus of a trial should be on patients who really need it. And those

patients these days in marrow transplant centers and in most intensive cancer centers are on difficult to treat organisms, particularly yeast and molds and some resistant bacteria. We believe in contrast to earlier eras that the best way to proceed would be with cooperation, a multi-center trial. I showed you reports of small, relatively inconclusive trials, and part of the problem was how they were conducted. And finally, you need to do them with some standard approach to the patient care, the other aspects of patient care, in order to be certain about what you see.

In terms of trial design then, the best ideas are to use people who have marrows that are expected not to recover quickly, that is, they have received aggressive chemotherapy or transplant. We believe that this is an applicable approach to patients with neutropenia at present. There may be other ideas, but that should be the focus. And as I mentioned, fungal infections and people preferentially chosen to be not demonstrated to be alloimmunized before transfusion support is given based upon much evidence that you can alloimmunize somebody and not get a response. And the basic idea in a randomized trial should compare if patients have fungal infections or bacterial infections that

they receive a standard therapy, a standard therapy plus neutrophils procured in what I would say is a modern way.

Now there are many issues, and what I said may make it sound simple, but I assure you it is not. That is, we are still studying issues around mobilization strategies. How much difference does it make about which drugs and how much you give and when you give them. There are issues still about the quality of cells. I have said that cytokines affect cell formation and function, and that diversity of effects needs to be considered in terms of the actual trial design. There are lots of issues related to donor willingness and safety. It is amazing in this country the diversity in terms of the willingness of people to give blood. Reasons that are very complex. And if you think about another layer of complexity, that is accepting the idea of being treated with a drug before you give blood, you can imagine that there are many aspects of this to be considered if a trial is to be conducted well and conducted safely. There are many issues, some of which will come out today, about recipient benefits and risk, and then there is the question of having in a trial design good, clear, acceptable evidence of therapeutic efficacy.

In terms of how this should be done, I believe and I think many do that neutrophils are there to kill microbes. And so a trial should be designed primarily to show an effect on microbes, that is, the clearance of infection. There are many secondary endpoints, though, that are important. Important socially, important for patient well-being, important for the people who pay for medicine in this country. So that one has to decide a trial with enough other information in it so that you can apply the results of a trial in the clinical and economic settings where we practice.

My final point in terms of general comments about a clinical trial comparing the past with the present is that we live in a very dynamic world. Look at the paper today, right? And in this particular field, we live in a world that is changing rapidly. I have said enough, I bet, to convince some of you, if you weren't already convinced, if you went home tonight and it was your mother or father or sister or brother and I offered you this, you would say, of course. And if I said you might not get it in a randomized trial, you would say, oh no, I don't want to participate. Because times have changed. This approach does show considerable promise. And I think that the window

of opportunity in clinical trials in this field, like in other fields, is relatively small. Because knowing as I do people around the world who work in this particular area, many people are taking this approach and taking it without really firm evidence that it is a clinical benefit. So the time for a clinical trial is relatively brief.

I am going to stop at that point. Again, it is great to be back at the NIH and thank you very much.

CHAIRPERSON HARVATH: That was really great. Thank you very much. It is a pleasure to introduce the next speaker, Dr. Thomas Price, who is also a Professor of Medicine at the University of Washington and the Director of Puget Sound Blood Center. He is going to speak to us about his experience of cytokine administration to normal granulocyte donors and some other really great information I think you are going to add as well. Thank you.

DR. PRICE: Thanks, Liana. Thanks to you and the organizers for inviting me here. If I could have the first slide. What I am going to do today is to share with you our experience, which is an ongoing experience, with a trial of neutrophil transfusion that we are doing in collaboration with

the people at the Fred Hutchinson Cancer Research Center involving patients that are undergoing bone marrow transplantation. And I would just like to tell you kind of where we are with this.

This slide you have seen before. It is to kind of remind you of the mobilization that we are talking about here. And as David said, we looked at these five different mobilization regimens here. The dotted lines are the ones that included dexamethasone in addition to the G-CSF, and as might be obvious from looking at this slide, in the trial that I am about to show you, we picked the one that we thought was going to give us the most granulocytes. So that is why the study that you will see and that I am going to talk about now involves the 600 mcg dose of G-CSF and also dexamethasone. Now whether it will turn out that there is really that much difference between these top two, we won't be able to say as a result of what I am going to tell you.

The other thing to note, of course, is that the timing in here is to suggest that doing this 12 hours before you collect the neutrophils would probably be the best time to do this. So this is what we aimed for, but keeping in mind with the logistics of when donors can actually show up and

when you can actually collect the cells. As you see, we don't always hit the 12 hours right on the mark.

The basic design of this ongoing study is shown in this slide here. This is sort of a Phase I/II study, as I said a collaborative study between the Blood Center -- this is the Puget Sound Blood Center, it is not a misprint for peripheral blood stem cells here -- and the Hutchinson Cancer Center. One of the wrinkles on this thing is that the design here is to use community donors. Now most of the studies that have been reported using G-CSF stimulated granulocyte donors have been when the donor has been a family member or friend of the patient, a fairly captive person that you can lasso and do this to. The idea here was to see if we could supply granulocytes to patients as they needed them using community donors. These are donors that are just ordinary blood donors who have volunteered to be, for the most part, platelet donors or to be apheresis donors for patients that they don't even know. Could we involve them in such a process?

As I said, the dose mobilization was to give them 600 mcg of G-CSF and 8 mg of dexamethasone. This was done as close as possible to the 12 hours prior to the collection procedure. The collection procedure itself was routine. We

used a COBE spectra machine. We used the high molecular weight hydroxyethyl starch, the hetastarch, as the red cell sedimenting agent, and we processed 10 liters of blood for these collections.

Now the patients were all patients on the bone marrow transplant ward and the Seattle transplant ward at the Hutchinson Cancer Center. Most of these, as you will see, were patients who had already received a transplant, although there were a few patients in there who were pre-transplant. They are all neutropenic. The idea was to limit this to people who had 100 neutrophils or less. And they were people with documented fungal or resistant bacterial infections.

Now the original plan or the goal of this thing were these three things listed here. One of the things we wanted to do was to evaluate the feasibility of using community donors. We started out by just calling some pheresis donor up and saying how about coming in and getting a shot of G-CSF. We had no idea how easy it was going to be to convince people to do this and whether we could basically supply with any kind of regulatory these components. We also wanted to see what we actually could get in neutrophil yields by using this sort of

a mobilization strategy. The slide before was, as David said, just some normal guys that we gave these things to and did some blood counts on. The proof of the pudding was what we would actually get when we hooked them up to a machine.

And finally, we wanted to determine what the hematologic effects were going to be in the recipients. What was going to happen to them in terms of their counts and in terms of where these cells went. It would have been nice to make some sort of clinical determination of whether this was efficacious in the recipients, and we were going to look at that. But right from the beginning we knew we weren't going to have enough patients probably to really make a determination that was convincing of clinical efficacy.

Let me turn a little bit to the community donor recruitment business. The way this worked was we have got this pool of 4,000 or 5,000 people who have signed up to be pheresis donors. For the most part, these people are platelet donors, but they are also subject to being called for a granulocyte collection which traditionally has involved taking some prednisone as a stimulating agent. The idea was that this regular donor list was that the donor would be contacted by the regular

pheresis scheduler, somebody that they would almost know because they talk with them fairly frequently, and be informed that we had a study going that we thought was likely to be able to improve the product, but it did involve them taking a drug and would they be interested in participating. If they were, then they were scheduled for a donation, but they were also then put in contact with the study nurse coordinating this study who went over the study in detail with them and basically went through the informed consent procedures as to what this was all about.

Then what happens is the 12-hour ahead of time visit, the donor comes in to one of the Blood Center's fixed sites. We have five sites scattered around the Seattle area and the donor could come to any one of these sites 12 hours before. Now as a practical matter what this meant is that we tried to shoot for 12 hours, but as a practical matter it was somewhere between 8 and 16 hours ahead of the scheduled leukopheresis. What this means is that you can't just do a leukopheresis at any time. The leukopheresis had to be either scheduled at the crack of dawn so that the donor could come in the evening before at a reasonable hour -- come in at dinner time, you know 7:00 or

8:00 at night, and then we could do an early morning collection. Or alternatively, the donor could come in at the crack of dawn for the pre-visit and have the blood drawn at 7:00 in the morning and then we would be able to do a leukopheresis procedure in the 5:00 in the afternoon sort of range. But it does sort of limit you because you can't do a collection procedure at 1:00 in the afternoon because there is no right timing for the donor to come in for the ahead of time visit.

Anyway, when they do come in, the consent form is signed. They go through a preliminary donor screening, just to make sure that there are no surprises there and 12 hours later we are not going to find out that the guy had hepatitis last year. We also draw blood for the routine things. We draw blood for a CBC and also for the ordinary testing -- for the ABO, the Rh antibody screen and for infectious disease testing. And then, of course, the G-CSF is administered subcutaneously and the dexamethasone is given for the donor to take.

Now since this is an FDA sponsored conference, I did want to make one what I think is important point about the infectious disease testing. What we did in this study is considered

that the testing that we did on the sample obtained 12 hours prior to the leukopheresis, we considered this to be the testing of record for the collection. Now for those of you who are in blood banking, this is not the usual FDA approach to things. The usual FDA approach is to say that you have got to draw the blood sample that you are going to do for the testing actually at the time that you are collecting the blood component. Now I think it is important, though, that we be allowed to do this as I have described it here and count this as the testing of record because of basically what David was saying about the storage capabilities of these cells. With the current techniques, the neutrophil integrity is likely to be compromised if we store it waiting for these tests. With the more sophisticated testing -- I mean, stuff that we have done in the past basically has shown that if you store cells for 24 hours, in terms of the cell's ability to localize to an inflammatory site, the cell loses about 75 percent of its activity at 24 hours. So it is very important, at least now, to give these cells as soon as possible after collection and we can't really wait until all the testing is done. And this is getting worse because the time for testing keeps getting longer. When PCR comes along, it is even

going to be worse. Also, it is not -- you know, the testing lab is not necessarily right next door these days to the place that you are drawing this blood. It may have to be sent off to a different contract place that is doing the testing. So this timing gets worse and these things do not store well. So I guess I am making the pitch that should the FDA decide they want to write some rules for this, that it would be very important to be allowed to have the testing of record be this sample that we draw the day before, let's say.

Now it may turn out, to be optimistic perhaps, that one of the effects of G-CSF will be that we will be able to store these cells better. But there is really no data on that in vivo yet. So that is just totally an unknown right now.

Okay, well what happened? We had as part of this -- what I am going to tell you about so far is our experience with 19 patients that we were trying to provide granulocyte support for. If we started at the time that these patients were identified up until the time that granulocytes weren't needed any more, there were 233 slots that had everything gone swimmingly we would have had a collection for each of these slots. As I said, we have about 4,500 donors in our pool that we have

available to call. One of the things that surprised us, I guess, was that when we did contact these donors and said how would you like to do something kind of experimental and get a subcutaneous injection of a drug, about two-thirds of them said fine, I will be right in. Now because of various logistic things and part of having to deal with this timing that I am telling you about and the time slots that had to be available and the donor had to be able to fit, in fact we only succeeded about 75 percent of the time in getting somebody actually when we wanted them. That was more of a logistic problem than it was a problem of not being able to find a donor.

What I am going to report to you now is the results of 175 collections with this stimulation here, this 600 of G-CSF and 8 of dexamethasone. A little bit about donor side effects. You have heard many times and those of you who were here yesterday heard again the story of donor side effects from G-CSF. The experience we have had at the Blood Center has been similar to all of this. Most of the donors experienced some side effects from this pre-stimulation. Mild to moderate in the vast majority of donors. With these 175 donors, 40 percent experienced some sort of bone pain, 30 percent

headache, 30 percent insomnia, probably more due to the dexamethasone than to the G-CSF. About a third of these patients or a quarter to a third of the patients had no side effect at all.

Now the severity of these side effects I think you can judge by the fact that of the donors that donated, when we asked them later how big of a deal this was, 98 percent of them said that they would be more than willing to come back and do it again.

What was the experience in how much we actually got. The donors neutrophil count at the time prior to getting the G-CSF on the sample that we drew 12 hours ahead of time was normal. It averaged 3,700 with this sort of range. The time interval between getting G-CSF and the beginning of the collection averaged 13 hours. As you can see here, it varied with an extreme for 5 and 23 hours. The donor neutrophil count right before the collection was almost 31,000, varying here between 14,000 and 56,000. This is the neutrophil count now and not the white count. And the number of neutrophils that we got averaged 82 billion. It ranged between 24 billion and 144 billion. Now just to remind you of the numbers that David showed you before, the traditional neutrophil yield that is

obtained by modern cell separators using corticosteroids alone as a stimulus is usually in the 20 to 30 billion range. So you can see here that this now is two to three times the yield that you normally get without using G-CSF.

Now this slide here shows you the relationship between what the donor's neutrophil count is right before the pheresis procedure and what we get in terms of the yield going all the way up to -- I can't really read that but it looks like 160 billion cells there. This, again, is neutrophils and not white blood cells. I think you can see that there is a little bit of scatter here, but there is clearly a pretty linear relationship between what the donor's neutrophil count is and what you get out of this. It nicely extrapolates down to zero. What you see here actually as the small dots are the 175 dots representing the donors that I am telling you about. The heavier dots there are another roughly 20 collections that we have done where we used only 600 mcg of G-CSF and did not give the donor dexamethasone. I think you can see that you basically get a lower neutrophil count and you get less yield. Remember that the average neutrophil count in the 175 was about 30,000. The average for the G-CSF alone is about 22,000. The

average yield is 82 billion for the combination and about 56 billion for the G-CSF alone. But the interesting point is that the G-CSF alone dots, the big dots, appear to be in the same continuum as the other dots. It is just a matter of how high you get the count. So it looks like this is kind of a validation of the idea that the higher you get the count, the more cells you are going to get. It is sort of a no-brainer. But if we could figure out a way to routinely get the cell count up to 60,000, we might be able to get a lot more cells. We might get the average yield up to 160 billion.

Now who are the recipients here? These are the 19 patients that we gave these cells to. 15 of them had had a bone marrow transplantation and 4 of them, as I said before, were pre-transplant. 16 of these patients had a fungal infection, 8 fungemia and 8 an invasive infection. Most of these are Aspergillus infections, either pulmonary or sinus infections. And 4 of the patients had resistant bacteremias. You can see that this adds up to 20, which means that one of the patients had two infections.

Another item which I think is important to note about these guys is that in general this population was not an alloimmunized population. We

did HLA antibody screens at the beginning of the transfusion support on all these folks and the screen was negative in 15 of these 19 patients. There was a little positivity, but not very much. The PRAs were less than 8 percent in 4 of them. So none of these patients was highly alloimmunized to begin with and most of them had no evidence of alloimmunization.

What did we see in the patients in terms of the hematologic results? There was an average of 8.6 transfusions per patient. It ranged from 1 to 25 transfusions. You have already seen that the average dose delivered was 82 billion cells. Now this is what happened to the patient's neutrophil count. I have listed two things here. One is the one-hour increment, that is, comparing the neutrophil count one hour after the transfusion with the count immediately prior to the transfusion. You can see here that the average was about 2,600. And as David mentioned, this is in marked contrast to the usual experience with granulocyte transfusions where one didn't see any increment and we always used to say, well, that is because they are all doing what they are supposed to do and going to the site of infection. But in this situation, you actually do see a substantial increment in the

neutrophils. And you can see that this varies from one guy who actually had a negative increment who had a fairly high count to begin with pre-transfusion to a very high neutrophil increment.

The other thing to note is that these cells stick around. They do drop off as the day goes on, but if you do a count the next morning, the average count in these people was 2,600. This is just a coincidence that these happen to be the same number -- varying anywhere from nothing to 15,000. So that you can see on the average we are taking patients who begin severely neutropenic and we can, again on average, convert them from somebody severely neutropenic to somebody who has a sustained neutrophil count which is normal or near normal. Now I will also take you back to the other slide David showed you of the sequential days and the sawtooth sort of thing where if you would actually pull out this next AM count, of course you start out at zero in these patients and after the first -- it goes up for one day and comes back down again but not quite back down to where it started from, and then on day two you get it up a little higher and you can sawtooth this thing up. So that the general experience is after a few days, the patient is

running a neutrophil count that is often in the normal range.

Now you will note some low numbers here, though. Two of these patients got no increments. So this is the average, but a couple of them got no increment at all.

Now the next thing here is what we are calling the buccal neutrophil count. This is kind of a crude way of determining whether these cells are capable of leaving the circulation and getting to the tissue sites where it is important that they do their work. And what we do to this thing is have the patient take 24 cc of saline into their mouth, swish it around, spit it into a can, and then by staining the cells and counting them, we can count how many neutrophils are in the guy's spit. What we find is that when we did this before we started the transfusion support, basically this is in millions, the average is .01 with this sort of a range here. Post-transfusion -- and generally we made these measurements the next morning after the transfusion -- you can see that on average there were about a half a million cells in there with this sort of a range. Now if we were -- just to give you an idea of the normal numbers -- if we were to do this same little test in everybody in the room here, the

average amount of neutrophils in a normal person is about a half a million. So these guys on average were going from nothing up to where the amount of neutrophils in their mouthwash was normal. So these cells are capable -- they don't just circulate, they are capable of extravascular migration and getting supposedly to where they are supposed to go.

Now again you can see that there is a range here. In fact, there turned out to be a correlation between this. The guys that got no increment in the blood also got no increment in the buccal neutrophils. This is what you would expect, I guess, but it sort of validates that maybe we really are measuring here something that means something.

What happened in terms of the side effects? I think it has been mentioned here earlier that one of the concerns of giving much larger doses of neutrophils, particularly neutrophils that have been primed by G-CSF, the early concern was that this might give an awful -- this might sort of exaggerate transfusion reactions and might exaggerate in particular pulmonary transfusion reactions and be a dangerous thing to do. So we are looking here then at the 175 collections, but only 165 of those ended up being transfused. If we look

at the traditional sorts of things here like chills and fever, you can see that in about 7 percent of the transfusions, one saw that patients got chills and some fever. This meant that in these 19 patients about a third of them at one time or another in one or more of their transfusions had this experience. These were mostly mild to moderate, in fact. And actually what tended to happen was these patients would then on subsequent transfusions be premedicated with Tylenol or something like this and most of the time they did not recur. So they tended to be things that were easily handled by the usual pre-medications you might give. There were an unusual number or a low number of itching-hives type reactions.

Now the other thing we do since we were particularly concerned about the pulmonary reactions, is we measured oxygen saturation by oximetry prior to the transfusion and after the transfusion. You can see here that the baseline oxygen saturation was about 95 percent. It varied between 61 and 100 percent. Some of these patients, particularly the guys with pulmonary Aspergillus, might not start out with a normal oxygen saturation. On average, the change was basically not existent. But if you looked at these individual things, of

these 165 transfusions, the oxygen saturation decreased by 4 percent or more in only 11 of the transfusions and by 6 or more in only three of the transfusions, and in those three it actually ended up below 90. The important clinical point to make is that in no transfusion of these 165 was it ever the clinician's view that something had happened. These were just kind of measurements that were made, but there was no obvious pulmonary deterioration that was attributed to the transfusion. So the fear that suddenly we could have set ourselves up for a real dangerous transfusion reaction doesn't appear to have happened. It is important to remember, though, that these patients are not highly alloimmunized people. It is not that kind of set of patients.

Now a little bit about the HLA compatibility. And you can tell by the fact that it is a cheaper looking slide that this is preliminary data. What we did is we obtained serum samples on all of these patients before we gave the first transfusion and then weekly thereafter until the patient was off-study. We also, every time a donor came in, obtained lymphocytes from that donor and froze them. So that after we are done with the patient, we can retrospectively come back and in

sort of a batch run all those serum samples against all those lymphocytes and basically do a lymphocytotoxic cross-match for each transfusion that was given. We have those results available on 12 of the 19 patients so far. What happened was if we looked at all these serial blood samples, there appeared to be an antibody to one or more of the donors in 4 of these 19 patients. In other words -- well, I should say 12 -- the people that are finished. In 8 of these guys, it was clean. The lymphocytotoxic cross-match was negative in everything. But in 4 of them, there was a reaction to one or more of the donors, and it turned out to be 14 potentially incompatible transfusions. Now I say potentially because some of these things were situations in which a late serum sample might show a reaction to an early donor, but whether or not the antibody was there when that donor was given, that might not have happened. But for the purposes of this analysis, I would have to assume that if it ever happened, it might have been there at the time of the transfusion.

Well, if you look at these 14 potentially incompatibles, what you find is that of the 14, none of them was associated with chills and fever in the patient. In one of them, there was

somewhat of a decrease in the oxygen saturation. The average neutrophil increment at one hour was 2,300 and the next A.M. count was 2,200. So that if you will remember the overall group, these were both 2,600. Just on the service of it very preliminarily, it doesn't look like these HLA antibodies that showed up during the course of this really had any influence on the transfusion reactions or the hematologic results of the transfusion.

Well, how about what finally happened to the patients. The reasons for discontinuing the transfusions are listed here. In 7 of these 19 patients, we stopped because the patient's neutrophil count was high on its own or the patient had grafted. In 3 of them it was stopped because the infection appeared to be gone. And in 9 of them it was stopped either in 6 because the clinical situation was determined by the clinician to be futile and support was withdrawn, and of course the ultimate futile situation when the patient died.

If you take all of these patients, 9 of the 16 survived until engraftment and 8 of the 19 cleared the infection. If we sort this out by the kinds of infection, about half of the patients with the fungal infections, whether it was either

fungemia or Aspergillus, survived until engraftment. That was true of everybody with bacteremia. In terms of clearing the infection, about half of the fungemia patients cleared the infection, nobody with Aspergillus was thought to have cleared the infection and all of the bacteremias were thought to clear the infection.

Now what to make of this. This is one of these things that you can read as the glass is half full or the glass is half empty, I think. I would say that the general impression of the clinicians on the ward was to be impressed that this was probably useful therapy. There were a number of these patients that anecdotally were people that they thought normally would have done very badly who ended up clearing the infection or surviving longer than they thought they otherwise would have, but that is obviously just a clinical impression anecdote style and may or may not hold up.

So in summary so far, I think we can say that perhaps surprisingly that community apheresis donors are fairly easily recruited for G-CSF stimulation, that such stimulation in normal donors results in marked neutrophilia and greatly increased neutrophil yields, that when you transfuse these concentrates into patients, this can result in

normal or near normal neutrophil counts in the recipients with migration of transfused cells to extravascular sites, and that although the clinical impressions are sometimes impressive and the clinicians can be convinced that they are really being useful, I think we can't really say that based on these numbers and that we really are going to need control trials to assess the clinical efficacy. Thanks very much.

CHAIRPERSON HARVATH: Thank you very much. That was very interesting data. Before taking the break, Dr. Epstein, who is the Office of Blood Director in the Center for Biologics was sitting next to me and we were talking about the question that you had asked of the FDA. So before he has to leave, I would like Dr. Epstein to address your question and then we will take our break and then we will assemble a panel after the last two speakers of the morning session.

DR. EPSTEIN: Thank you very much, Liana. Just a brief comment. The current regulations require the donor sample to be obtained on the day of collection, and we can interpret that broadly to be within 24 hours, certainly encompassing 12 hours. There is no requirement that the sample tested be integral to collection,

although clearly we prefer that. Additionally, if it proves to be infeasible to test within 24 hours, the regulations provide for the possibility of exceptions to the regulations, and you simply have to request an exemption. It is under 21 C.F.R. 640.120. So I think that there really isn't a regulatory obstacle to doing what is scientifically and medically appropriate. We just have to be in the right dialogue.

CHAIRPERSON HARVATH: Okay. On the panel discussion, the presentations this morning also raised some very interesting questions that I think would be good to ask of the panel and get all of your feedback on for the experiences you have had. One of the things I think that will be helpful to us will be your collective experience on whether this is going to be a major obstacle and what your experience is with the testing of the products. Also, there was early report in the literature that perhaps some cytokines may alter some of the test results, and I know that we have heard this, and I think it would be very interesting to pose that question to those of you who have been collecting these products and actually performing the routine tests on your donors. So it would be something very interesting to hear of all the speakers.

I would like to give everyone an opportunity to take a 15 minute break and we will come back here to begin the second part of the morning session at 10:00.

(Whereupon, at 9:44 a.m. off the record until 10:10 a.m.)

CHAIRPERSON HARVATH: We are going to try and get started. Our next speaker, Dr. Dan Ambruso, is a Professor of Pediatrics and Associate Professor of Pathology at the University of Colorado Health Sciences Center, and he is the Associate Medical Director of Bonfils Blood Center. It is a pleasure for me to introduce Dan, and he is going to talk to you about his experience regarding the functional properties of granulocytes that he studied from donors after G-CSF administration. Dan?

DR. AMBRUSO: Thank you, Liana. It is a pleasure to be here this morning. I am happy to be involved in this workshop. I am going to present to you some information that we have on normal volunteers who received G-CSF, and I will say at the outset that Tom Price has talked about response that his donors had with a single dose of G-CSF, and I am going to talk about a project that we were involved with where our patients received five doses of G-CSF

and we looked before and after the administration and tried to focus on neutrophil function.

This seems initially a little bit farther away from the practical aspects of blood donors for granulocytes where a single dose might be more practical. On the other hand, I am sure all of you are aware of the fact, and this was brought up at the meeting yesterday and it has been brought out in the literature recently that there may be a number of paradigms including multiple dose administration of G-CSF to not only collect stem cells but also granulocyte support for these patients. So I think this information has relevance to granulocyte collections.

The objective of this talk, as I stated, is to review detailed studies of neutrophil functional capacity obtained during G-CSF administration, multiple dose administration. I would also, if there is some time at the end, present some preliminary findings of functional capacity of neutrophils stored in the presence or absence of G-CSF. That was part of the study as well. And then I would comment on areas that we think need further study.

The previous two speakers covered this part of the talk and I don't need to go into this in

detail. Everyone knows that the effects of G-CSF include increased numbers of mature neutrophils enhanced, and I put that with a question mark because I am in agreement and I will show you data to substantiate this, that neutrophils are really different. But the concept of a neutrophil which is red hot and angry and ready to explode and kill anything in its path is probably not what we get when we mobilize and we treat patients with G-CSF. And one of the other interesting and important effects of G-CSF is prolongation of time to apoptosis and its effect on program cell death.

Our clinical protocol is summarized here. We had healthy adult volunteers and in subsequent slides I will call these patients. There were 9 males and 5 females. We administered G-CSF at a dose of 10 mcg per kilo subcutaneously for 7 days. Some of these patients were part of a control trial for the ACTG stem cell mobilization study. We looked and took peripheral samples or samples of peripheral blood before the first dose and after the fifth dose of G-CSF. In the studies, when you look at the data, day 0 is the first day. So we actually sampled before the day 0 dose, and day 4 is when they received the fifth dose of G-CSF. In 8 of these subjects, we completed granulocyte collections

after the fifth dose. The collections were completed using the COBE Spectra with hetastarch and the granulocyte products that we obtained were stored in the absence or presence of an added G-CSF 25 nanograms per ml at 22 to 24 degrees Centigrade in a stationary state. Samples were removed from these products at 24 and in some cases 48 hours of storage for analysis.

I will start out with some of the adverse events. To summarize, as has been other people's experience, there were very few adverse events in this administration. All of our patients had a mild headache and bone pain. We would rate it as grade 1 to 2. One patient dropped out of the protocol at the end of the fifth day or fifth dose in apheresis, but all of the rest had much milder problems. It usually peaked by day three of administration and usually treated well with either ibuprofen or acetaminophen with resolution of the symptoms. All of the symptoms completely resolved within 24 to 48 hours of discontinuing G-CSF.

Just a few words about the quantitative response. As with other studies that have looked at mobilization of neutrophils with G-CSF, we saw a marked increase in the leukocyte count from a mean of 4,870 per microliter up to almost 32,000, an

almost tenfold increase in the absolute neutrophil count, and a marked increase in the percentage of band forms in these individuals.

Now I show this slide as a prelude to reviewing the functional data on the neutrophils. Most of you don't need a lecture on neutrophil function, but I do this to give you a perspective and a focus on how we organized our function studies. As you know, neutrophils travel in the laminar flow of the blood stream until they identify an area of inflammation, exhibiting a rolling behavior at first and then finally sit down with firm adherence, diapedese through the endothelial barrier and move towards the area of infection or inflammation. Once they get there, they ingest the microorganisms, which does two things. Associated with ingestion is activation of the respiratory burst, the neutrophil NADPH to oxidase enzyme system, which is responsible for initiation of production of oxygen radicals and is associated with oxygen dependent killing. In addition, there is a variety of contents in the granules which then are released into the phagolysosome which affect oxygen independent killing.

So we essentially focused into two general groups, those kinds of function and

biochemical parameters that are related to adhesion and motility, and those that are related to microbicidal activity and degranulation or the status of the granules.

I present this also because again of the concern that over the past 10 years or so, somehow we have the concept that the neutrophil that is produced under the influence of G-CSF, this is not only for donors but certainly for patients who get G-CSF, is a neutrophil that is charged and ready to go. And my concern is, and this certainly has been presented by Dr. Price in the last talk -- my concern is that in fact if this were so, we would be in big trouble. When the neutrophil is able to get to the site of infection and to eliminate the organisms, that is one issue. If the neutrophil is charged on the endothelial surface and is activated on the endothelial surface, you get excessive inflammation and probably you get -- this is responsible for a lot of multi-organ failure syndromes which we see certainly in the lung and perhaps other organs. So my concern about this has always been that if the neutrophils are so charged, we are going to be putting patients at risk for these multi-organ failure syndromes. Certainly that is not borne up in the patients who have gotten

granulocyte transfusions, but perhaps the patients who have received granulocyte transfusions or G-CSF for clinical indications.

The first thing we did was to look at chemotaxis. This was done in a modified Boydian chamber. The results are summarized here. I hope that is in focus for all of you. What you can see is that we looked at non-directed migration towards buffer and we looked at migration with zymosan activated serum, which is essentially C5A. The open bars are the 0 values and the closed bars are day 4 values for controls and for the patients, that is, the volunteers who received G-CSF. And what you can see here is a marked decrease in the motility in this Boydian chamber assay in response to zymosan activated serum. There is a mild effect, although this isn't statistically significant, in terms of directed migration. So there seems to be in the neutrophils that are circulating after the fifth dose of G-CSF administration, there appears to be a decrease in cell motility.

We looked at two other parameters that are related to cell motility and might be a reason for the reduced motility. One is to look at the expression of CD11B, which is one of the major adhesion proteins for the neutrophil. And we looked

at the expression of CD11B in response to 4 ball ester at the concentration noted here, FMLP, which is a bacterial tripeptide and platelet activating factor. This slide summarizes the results for day 0 patients and controls and day 4 patients and controls. And what you can see is that expressed as a ratio, the stimulated up-regulation of CD11B was no different in the treated patients. In addition, what I don't have here was the baseline expression of CD11B, which was not increased in the patients on day 4 of treatment. So we could not blame the decrease in motility on a change in perhaps an up-regulation in CD11B.

One of the other biochemical correlates related to motility is F-actin assembly and one can measure this with a dye MBD felacydin. One of the other questions we asked was whether there was something that was different in the modal apparatus of the cells. So again we looked at the two groups, the controls and patients on day 0 and day 4, and this is a lot of data. The important thing is to look here. This is again an expression of mean channel fluorescence in unstimulated cells or cells that are treated with 10^{-7} molar FMLP. And what you can see is a decrease in F-actin assembly that is

statistically significant and we think probably practically significant too.

So in fact one of the things that we were able to find then was a decrease in motility and an associated decrease in F-actin. One of the other things we wanted to look at was channels in cytosolic calcium. This ends up being very important in motility and ingestion and in most receptor mediated functions of the cell. In these experiments what we did was to load the cells with a fluorescent indicator, binding calcium Endol-1, and we treated the cells with FMLP. This is a plot of the results for one patient. The neutrophils that were collected on day 0 were assayed and the calcium flux was followed in response to FMLP and the same patient on day 4. What you can see on day 4 is a marked increase in the total flux in the cells. The onset and initial rate are the same for day 0 and day 4, but this increase is two to three-fold. If we look at all of the patients now, and this slide summarizes results for all patients, looking at cytosolic calcium, and what I have here is the response of cytosolic calcium to FMLP 10^{-7} molar and also platelet activating factor. There are two columns for each stimulus. The first column reflects the baseline level of calcium and the

second the peak calcium flux. This is in micromolar concentration. What you can see -- the important thing -- again, a lot of numbers -- the important thing is to look at the second and fourth column at the bottom. This is the results for day 4. One can see a marked increase in all of the patients. This is a two- to three-fold increase of cytosolic calcium.

This enhancement in calcium flux in the cells in response to the specific stimuli, we are still not sure exactly what that means and the importance and relevance of this to the chemotaxis and perhaps other activities is not clear at this point.

So the next group or classification of studies that we did is spectracidal activity. This is a standard bacteriocidal assay. In these studies, this summarizes studies for the normal controls and patients on day 0 which are included in the dots and in the squares are patients on day 4. In this assay, there is a 1 to 1 ratio of bacteria to cells, and the bacteria is Staph aureus. This is done in the presence of 10 percent normal pooled serum. What you can see is killing that is equivalent to control in the patient on day 4.

Perhaps there is a suggestion of attenuation of killing, but this is not statistically significant.

We looked at this in a little bit more detail because neutrophils when they first get to an area of inflammation have probably a lot more bacteria to phagocytose and kill than just one each. In addition, one can see in a variety of patient disorders a mild killing defect. So we wanted to stress the system and we did a killing assay with a ratio of 10 bacteria to 1 neutrophil. This is summarized on the next slide. What you can see is that at 30 minutes and at 90 minutes, there is a statistically different and I think practically different percent killing in this assay. So perhaps there is a mild defect, and I would underscore that -- I would say a mild defect in killing and it may or may not be significant.

We looked in detail at the respiratory burst and the oxidase activity. And I am going to go through in the next two slides looking at the respiratory burst measured as cytochrome C reduction in response to a variety of different agonists. The first one we used was FMLP, a chemotractant which at a little bit higher dose than used in chemotaxis will activate the oxidase. On this plot you see the control and the patients on day 0 and day 4. What

you can see here is an increase in the FMLP response in patients on day 4 compared to controls. This seems to parallel what was found in vitro and also other studies that have been done, studies that Dr. Dale presented earlier this morning.

When we look at a variety of other agonists to try to define or get a complete picture of the oxidase, we see some divergence in the results. These are results for host cell superoxide activity production with 4 ball ester. As we can see here, this is day 0 control and patient and this is day 4 control and patient. We see a marked depression in the PMA response. If you look at another stimulation sequence, and in this sequence we try to look at priming of the cells and we essentially prime the cells or incubate the cells with platelet activating factor for three minutes and then come back and look at the response to FMLP. This usually gives us kind of the maximum respiratory burst. This is even a stronger set of agonists than PMA or most other agonists that you can use to look at the respiratory burst. What you can see is again a decrease or an attenuation of the production of superoxide in the intact cells with this stimulus.

Now if you look at a third stimulus, which is opsonized zymosan, which is essentially a phagocytic stimulus which is coated with complement and so ingestion is most likely by complement and C3BI receptors, what one sees is no difference in the respiratory burst. This is probably a more physiologic stimulus. So you see a divergent set of reactivity that shadow or characterize the respiratory burst. Some are increased, some are low, and some are normal.

We looked very carefully at oxidase components and this would be -- I am not going to show you all the data, but this would be of interest to individuals who are looking at or are interested in the oxidase itself. What we found on these cells when we looked at subcellular fractions, we found increased amounts of cytochrome B558 in the plasma membrane and normal contents of the cytosolic oxidase components, the P47-phox, the P67-phox and the P40-phox. So the oxidase itself seems to be intact, but we seem to have to certain kinds of stimuli a decreased response, which would suggest that it is other systems perhaps than the structural oxidase proteins that are affected.

One of the other things that we did was to look at granular marker proteins in order just to

define the status of granules in these cells. As Dr. Dale suggested earlier, these cells look different. If you do EMs, you have a sense looking at the EMs that the granules are not quite the same and the granule compartments are not the same. So we looked at this and looked at alkaline phosphatase, which is increase, which everybody would expect and which has been really well documented as an effect of G-CSF on neutrophils. Myeloperoxidase seems to be normal. The specific granule marker, lactoferrin, seems to be decreased. The question that is raised by this data as to whether there is a defect in the specific granules themselves and their production, when we looked at cytochrome B, the content of cytochrome B was actually normal to increased. So this needs to be looked at a little bit more carefully. I am not sure at this point that we can say that there is a decrease in specific granules, but there appears to be a decrease in specific granule content in some proteins. And that may certainly have some functional impact on the cell.

One interesting side note, and that is that we have saved now cell lysates and subcellular fragments of plasma in the membrane and granules, is to look at also to save RNA, and the question is

what does RNA do to some of the genes and some of the proteins in the cell. The interesting observation that we made is that when one looks at the cells that are collected on day 4, there is a much larger amount of RNA which can be extracted. This is roughly the RNA content for 10^8 cells. This is control day 0 and patient day 0 and control day 4 and patient day 4. You see almost a two-fold increase in RNA. In fact, when you store cells for 24 hours, you double the RNA again in looking at how much you can extract from the cells. This is very interesting. We are not sure what the significance of that is, but I think it is going to be an important clue to some of the defects that we are finding.

This is just kind of a mental break. I wanted to talk a little bit about apoptosis, because this is another area that we evaluated in this study with patients. These tests are done by looking morphologically. We take cells at the sampling times and we isolate them and we put them into culture with RPMI and fetal calf serum. Then at different times after that, up to 48 hours, we take little samples out and we evaluate them for the extent of apoptosis. Of course these are very labor intensive studies and people go crazy as they stay

up in the lab and try to get these things done. So our argument was, was this a-pop-tosis or apop-tosis. I mean, you get pretty crazy when you are doing these kinds of experiments. Dr. Levy, who is from Dublin, Ireland, was a fellow in my lab who was involved with most of the studies that you are seeing here. He had a different feeling and he would come out with his thick Irish accent and say, no, it is not any of those, it is really O'potosis.

Let me show you an example. This was a technique in which you use a double stain, preputium iodine and acridine orange. These are both non-apoptotic cells. You don't see any of the nuclear changes related to apoptosis. This is a dead cell and this is a live cell. This is a live apoptotic neutrophil. So on the basis of these morphologic features, we would characterize during the culture the percentage of cells that were apoptotic, and we could generate a graph, if you will. We called it the LT50. It probably should be the AT, the apoptosis time 50, but the time to 50 percent apoptosis. That is what is graphed here. What you can see is the dark bars are the patients at day 0 and day 4 with the controls. What you can see is that before G-CSF administration to the patients or in the control group, we see a time to 50 percent

apoptosis, live apoptosis, is somewhere around 17 hours. If you look at patients on day 4 after the fifth dose of G-CSF, what you see is the time to 50 percent apoptosis is prolonged to about 34 hours, it is doubled.

In other studies to the in vitro system, we added G-CSF to a dose of 25 nanograms per ml. What we saw is that these cells could respond further by prolonging their apoptosis. You will notice that this curve is now a different curve starting out at 30 hours and what we see is a prolongation of the control and patient day 0 and control day 4 cells to somewhere around 42 to 45 hours. And you see the patient day 4, which had received 5 doses of G-CSF, is prolonged even further. So the cells have, at least in vivo circulating that we can take out and culture and look at apoptosis, have something going on that prolongs the process to apoptosis and that these cells can be further manipulated by adding G-CSF in vitro to prolong that time to apototic death even further. So this is actually the good news about G-CSF. This is not doctor-assisted suicide of the cells, but in fact the reverse, that is, we can help prolong the lifespan of the cells. That has, I think, some implications perhaps for storage.

So in summary, administration of G-CSF for five consecutive days is well tolerated with, I think, minor problems and adverse events. Although I would echo Tom's comments about how are you going to get normal donors to accept any kind of discomfort which may be significant. And certainly we can see an enhanced number of mature neutrophils. I haven't shown you this, but I will present a summary slide in a minute where you certainly get robust huge numbers of granulocytes that you can collect by apheresis. Interestingly, the time of apoptosis is delayed and that the neutrophils themselves that are mobilized under the influence of G-CSF continuous administration for five days present a divergent pattern of functional characteristics but overall the function is not markedly enhanced. And we would further summarize that we think the effect of prolonged administration of G-CSF and the advantage to patients themselves may be related more to numbers and the effect on apoptosis and perhaps survival and not so much the enhanced functional characteristics.

Let me just summarize some of the results we have in storage. We can perhaps talk about this this afternoon in the discussion section and the poster session. But as I said, there were 8

products that we stored with this in the presence and absence of G-CSF. What we noticed is that looking at superoxide and intact neutrophils to all the stimuli that I mentioned before didn't seem to change much during the first 24 hours of storage and then it deteriorated gradually by 48 hours. It was about half of what you saw that I had presented with the time 0 studies. Most of the collected neutrophils remained viable, that is, greater than 98 percent viability of the neutrophils in storage, and were not apototic at that time. Their apototic rate was probably no different than what we found for the neutrophils that were collected right at the time or just before we had done the granulocyte collections, and that is the 34 hour time. So there seems to be, at least for 24 hours and it may be longer, this is something we need to look at a little bit more carefully, a viability and postponement of apoptosis. Chemotaxis, although it was deficient -- as I showed you, the day 4 data was deficient -- in storage this didn't get any worse, at least for the first 24 hours of storage. And when we looked at all these things and the addition of 25 nanograms per ml of G-CSF, we really didn't change these characteristics of the neutrophils. That is probably most likely that we didn't add

enough and that may need to be looked at a little bit more carefully. But there may not be really any additional effect that these cells have.

So what are considerations for future studies? First of all, I think more work needs to be done in defining standardized schemes for mobilization and collection. There may be reasons -- certainly one can collect granulocytes after a dose of G-CSF, but there may be reasons to look at multiple doses of G-CSF. There may be reasons to perhaps think about other cytokines as well. So this needs to be defined a little bit more clearly. It would be worthwhile to develop the optimal storage conditions for neutrophils to try to support normal function and survival for a little bit longer. Right now what we are doing, as Dr. Price has suggested -- and we have a difficult time in the blood bank because we get our donors in several days early and use that sample to allow the physicians to get the blood, that is, we can release it with that. We also have to do the processing on that sample that we collect. So this is really problematic. Obviously in terms of providing a product that has no storage time, if we could develop some, even for 48 hours, it would be very helpful in doing the collections. We really need to expand and develop

techniques for evaluating in vivo function of transfused granulocytes. To extend in vitro studies to evaluate this and to look at what their functions are in the patients is going to be very, very important. And, of course, clinical trials to document their efficacy, toxicity, and cost effectiveness are going to be important to complete in order to really revitalize this type of blood component. So with that I will stop.

CHAIRPERSON HARVATH: Thanks, Dan. The next speaker is Dr. Susan Leitman. Dr. Leitman is the Chief of the Clinical Services Section of the Department of Transfusion Medicine here at the NIH. She is going to talk to us about her experience with G-CSF mobilized granulocytes.

DR. LEITMAN: Thank you, Liana. And thank you for inviting me to speak at today's conference. This is a slide I made to entitle a talk at another conference on this topic, and I found that it applied well to the issues that we are bringing before the FDA perhaps in consideration of licensure of this product. And with growth factor mobilized granulocytes, is this an exciting or stimulating -- no pun intended -- new component or are we stuck with the same old problems.

I would like to remind all of us, as if we needed to be reminded, that despite nearly three decades of clinical experience and dozens of publications of observational or controlled studies, the FDA does not recognize granulocyte concentrates as an approved blood component. And from the very nice review we heard by Dr. Dale this morning, there are very good reasons for that non-recognition. This is my version of Ron Strauss's review from the Blood 1993 article reviewed by Dr. Dale this morning. I want to point out that the reason we can't find efficacy across all of the seven prospective, some randomized and some non-randomized studies, is in large part due to the choice of the subjects for the study, and thus in studies designed to determine clinical efficacy you have to choose patients in whom the mortality is estimated to be substantially above 60 percent. In those studies in which the survival was 60 percent or greater, no efficacy could be demonstrated. It was only when the mortality was quite high that efficacy could be demonstrated here, with mortality of percent surviving of 26, 15, and 36 percent. So in designing prospective trials, we have to choose the right population to study and, as Dr. Dale already

stated, have sufficient numbers to power the study or analysis.

I will talk just for a moment about dose because that has been covered very well so far this morning. To remind you that granulocyte apheresis yields without any donor preparation are in the range of .3 to .5 times 10^{10} cells. With the addition of hydroxyethyl starch, this is high molecular weight starch, that increases or that doubles to .5 to .9 times 10^{10}. When one uses some combination and some regimen of steroid administration plus starch, that again doubles to 1 to 2 times 10^{10}. I am going to diverge for a moment and say that granulocyte apheresis took a giant step backwards in the mid to late 1980's with the introduction of a new form of starch, pentastarch. Pentastarch is a less highly substituted amylopectin backbone, the same backbone that is in hetastarch, with an average molecular weight of 264 rather than 480,000, a significant reduction in the number of hydroxyethyl groups per glucose residue. What made pentastarch very attractive to blood bankers was the safety for the donor in that the 24-hour urinary excretion is much higher with pentastarch than hetastarch and the overall survival in blood is only 96 hours as opposed to 17 to 26 weeks with some

residual hetastarch remaining in a donor's body for substantial periods of time. So the entire blood banking field sort of moved to pentastarch rather than hetastarch in the late 1980's. But if you look at the studies of pentastarch, the efficacy of granulocyte apheresis or the efficiency of granulocyte apheresis and the yields were never prospectively compared to hetastarch. A fellow in our lab, Dr. John Lee, who is now with the FDA, did a very nice set of studies in the early 1990's where he looked at the comparison of pentastarch and hetastarch. Just to remind you, the granulocyte collection efficiency with apheresis devices, the GCE, varies directly in proportion to the donor's erythrocyte sedimentation rate. The more quickly the red cells sediment, the better the separation in the granulocyte layer and the more efficiently the machine can collect them. So with increasing donor sedimentation rates, there is an increasing granulocyte collection efficiency. What was not known at that time or not clearly defined was that hetastarch quadruples the donor's sedimentation rate in vivo and pentastarch increases it by one and a half to two-fold.

When John Lee prospectively compared in 72 apheresis donors a granulocyte apheresis

procedure using pentastarch and three months later a granulocyte apheresis procedure using hetastarch and looked at the granulocyte collection efficiencies, if they were the same the line of identity would be here shown by this dash blue line, and they were not the same. The granulocyte collection efficiency with hetastarch was substantially and significantly better than with pentastarch in all but three donors. If you look at the yield, not only the GCE, you see again here is the line of identity and with hetastarch the yields were always, except for three donors, substantially greater than with pentastarch. I will summarize this numerically on the next slide. So there is a 60 to 70 percent increase in granulocyte yields times 10^{10}. This is before G-CSF. These are all non-G-CSF mobilized donors. From 1.4 to 2.3 times 10^{10} in these 72 paired collections. Collection efficiency increases from 33 to 58 percent. With the publication of this study, I believe that most centers have returned to hetastarch.

At about the same time that Dr. Dale was giving his five college students various doses of G-CSF, we were giving 20 healthy apheresis donors under protocol three varying mobilization regimens. The donors underwent three leukopheresis procedures

each separated by at least four weeks. The three preparative regimens were nearly identical to what you have heard this morning -- dexamethasone 8 mg orally was given 12 hours prior to donation. We would tell our donors to take the dexamethasone about one hour after dinner, which is about 8 p.m., and that is 12 hours before they come to our apheresis center at 7:30 to 8:30 in the morning. We give them the next dose of dexamethasone to take home with them at the time of apheresis so that they don't have to come to a pharmacy to get the tablets. G-CSF we administered at a dose, a per kilogram dose of 5 mcg per kilogram, and we used Amgen's form of filgrastim, and we gave it subcutaneously between 16 to 24 hours prior to donation. And then in the third arm, they received both types of preparative drugs.

G-CSF comes commercially or is available commercially in two size vials, a 300 mcg vial and a 600 mcg vial, 300 per ml. So it is one ml in the first vial and 2 ml in the second vial, which is exactly why in the Seattle study they used either 300 or 600. Apparently the Government on GSA schedule gets a vial that contains 480 mcg, which is partly why we use 600 mcg per kilo, because that does not exceed one 480 mcg vial. So this is going

to be a little bit different in comparing our study to the Seattle studies, but probably not substantially so.

All our granulocyte procedures are performed using a Baxter CS-3000 plus apheresis device, continuous flow, two access sites needed. These are the parameters of leukopheresis. The two instrument chambers who blood flow rate is between 50 and 60 ml per minute. Our endpoint volume traditionally in the last 15 years of collecting granulocytes has been 7 liters. So for this study, we did not change that. You will note the Seattle protocol was 10 liters and other protocols vary from 7 to 12 liters processed. The anticoagulant is sodium citrate. The sedimenting agent is 6 percent hetastarch.

If you read the operating manual for performing this procedure on the CS-3000, it tells one to set the interface offset at 15. No one does that. In studies done 10 years ago along with the engineer who developed this device, Mr. Herb Cullis, it was found that an interface offset setting of 33 yields optimal efficiency of the procedure, and so most of us have been using an IO of 33 for the past decade, not what is in the operating manual.

These are the results of the three different regimens, dexa, G-CSF, and D+G. This is the peripheral blood polymorphonuclear count immediately prior to apheresis, and you can see that the addition of G-CSF or D+G increases the white cell polymorphonuclear cell count in the donor by 3.5 to 4.5 fold, very similar to what you saw this morning, from 6,000 to 21,000 to 29,000. Similarly, the product content increases 2.5 to 3.5 fold from 2.5 times 10^{10} with our traditional dexamethasone alone arm to 5 to 7.2 times 10^{10} with a combination of both. All of these comparisons are statistically significant at the .05 level for every comparison within groups.

Addition of dexamethasone to G-CSF alone resulted in a 43 percent increase in the granulocyte yield in the product. We also looked at granulocyte collection efficiency and our usual efficiencies, as you have seen in the previous slide, are in the range of the low 60 percent. Somewhat to our surprise, we found that the efficiency dropped by 10 percent when we added G to this regimen. When we spoke with the engineer who had designed this device, he told us that the machine was designed for donor counts of 10,000, not for total white counts of 30,000 to 35,000, and that was necessary probably

was a complicated change to the interval between spillovers and the duration of spillovers, for those of you used to this machine, and that we would eventually do that. But as a start, we simply increased the interface offset setting to 45, and I don't have those numbers right here because they haven't changed. Simply increasing the depth of penetration into the buffy coat layer as this machine collects the cells did not significantly increase the GCE. So I think further work needs to be done with this particular device in maximizing collection efficiencies.

One day, we just happened to have three of these products quite by accident in the processing area of the transfusion medicine department, and they looked so distinctly different that we took the opportunity to take a picture of them. This is the traditional dexamethasone stimulated product. It looks redder because the buffy coat layer is less thick. This is G-CSF alone with a thicker buffy coat, and this is the combination of G-CSF plus dexamethasone. This is just sedimentation on the counter top over the course of the six to eight hours between the end of collection and the time of transfusion. You can see the buffy coat layer sediment out. In our

institution, we start apheresis in all such donors on this protocol at 8:00 to 8:30 in the morning. The product comes off the machine by 10:30 in the morning, and we do same day transfusion transmitted infectious disease testing starting at 9:00 in the TTV laboratory. So we do same day testing although we are one of the very, very few institutions that I think can continue to do that today. So our products are transfused at 8:00 p.m. that day. They spend about 10 hours on the shelf and we saw the sedimentation within those 10 hours.

What is the effect on the product of the three different regimens other than in the polymorphonuclear leukocyte count? We always process the same volume. This is the volume without the anticoagulant added. So this is actually actual true blood volume processed. The machine is set to 7 liters but about 500 ml of that is anticoagulant. The product volume is set by the operator. We traditionally set it to be about 240 ml. The platelet content was identical across all three collection regimens and the red cell content was identical across all three collection regimens. Just to remind you, the mean red cell content is about 30 ml of packed cells, so a cross match is always necessary between donor and recipient.

This is a summary that I made a while ago and it is not updated to reflect the newer Seattle data, which we heard this morning and which came out in August. But it looks at the four published studies at that time of granulocyte apheresis yields following G-CSF containing regimens. The dose varied from 200 mcg per day to 5 mcg per kilo. Steroid use was variable. Starch use was variable, pentastarch or hetastarch. Volume processed was variable. And the yields at that time were in the range of 4 times 10^{10} or 40 times 10^{9}, except for our experience in which it was double and the current Seattle experience in which it is right about this number -- 82 is what we heard this morning. I think the difference here was in the steroid use. In these initial studies, we were always using steroids in our clinical study in products that were getting administered to patients and we were always using hetastarch, whereas the type of starch used and steroid use varied.

I show this slide because it wasn't quite presented in this way by the earlier speakers today. This is the adverse reactions to mobilization regimens in donors who have undergone all there preparative regimens. With dexamethasone alone, we have an n of 38. And 44 percent of donors

have symptoms and they are almost all universally related to hyperactivity -- insomnia, feeling wired, feeling restless, waking up at night several times. And a small proportion had flushing and this was commonly delayed. So they would call us from work later that day to say their colleagues told them their face and their ears were bright red. It could also even happen the next day after. So 56 percent of such donors on dexa alone did not have symptoms. With G-CSF alone, the same number, n equals 38. We had the usual 39 percent with bone pain, 26, a quarter, with headache, 24 percent with that sense of wiredness, and also 5 percent with flushing. Only 32 percent did not have any symptoms at all. With the combination, the instance of bone pain is a little bit higher. Headache is about the same. Insomnia is the same with dexamethasone alone. Some nausea. I forgot to mention the fatigue. 10 percent have fatigue whenever you give G-CSF and flushing, so that only 28 percent did not have symptoms.

We have enrolled 120 donors on this study, similar to what Dr. Price described this morning. We took them from our pedigreed platelet pheresis donor population. And as he told you this morning, when you approach these intensely

altruistic individuals with yet another regimen or a new product that may increase the potential for survival for critically ill patients with cancer and other serious illnesses, they are most eager to cooperate and be, if you will, on the cutting edge of transfusion medicine practice. Of all the reactions I described on the last slide, 15 percent were judged by the nurses or myself in asking the donors these questions to be severe and interacting with everyday activities of the donor. 8 of 76 of our first donors or 10 percent -- and this continues, it is now 10 out of 100 -- have requested discontinuation of G-CSF mobilized collections. The most common reason was they didn't like feeling as if they had the flu once a month -- or as one donor puts it, I am tired of aging from age 40 to age 80 overnight once a month. The other common reason was the inconvenience of coming to the blood bank twice, once the day before the injection and once to donate. Donor reactions were stereotypic. Mild reactions tended to become milder with further donations. An initial 10 percent of donors had no symptoms on subsequent G+D mobilization although they had had symptoms on their first occasion.

Now I would like to talk for the remaining time on patient outcomes. The results in

the first three patients that we transfused with G-CSF mobilized products were so dramatic that as my colleague Harvey Alta says, you should make slides of things that work before you find that they don't work. Make your slides quickly. So we made these of the first three patients. Our very first patient was a 55 kilo female with T cell large granulolymphocytic leukemia who had a sigmoid phlegmon due to diverticulitis. She had a bacterial process. She was extremely ill and toxic, persistent rigors, chills, fevers, unresponsive to antibiotics. The second patient was a larger male, 130 kilo, day 10 post a T cell depleted marrow allograft for myeloma with a systemic Aspergillus flavum infection. The third patient was a similar day 7 post T cell depleted marrow allograft for CML with a systemic fusarium infection.

Let me go back before I do that. What was dramatic in all these patients was the almost immediate response to the administration of these cells. This patient became afebrile for the first time the day of the granulocyte administration and remained afebrile until she actually recovered her own white count. Both of these patients were showering skin with new systemic fungal lesions on a daily or more often than daily basis. One could

watch the new lesions develop. New lesions stopped developing with the first granulocyte transfusion and old lesions resolved with subsequent granulocyte transfusions. This is the saw-tooth pattern you see when you look at the increment in ANC, absolute neutrophil count, with each subsequent granulocyte transfusion. These are the first two patients, who as I said had very dramatic responses. The first got a total of four granulocyte transfusions. Her absolute neutrophil count was 0, increased to 2, and as has been said by speakers this morning, remained elevated for the next 8 hours, which we had never seen before. So the next day she gets another increment of 2,000 and goes up to 4,000. By the next day, she gets another increment and goes up to 6,000. We do not collect on the weekends unless it is a very serious patient problem. So we did not collect on this day and she promptly dropped her neutrophil count at 30 hours. We gave her one more transfusion and then you can see her own cells recover.

This is the recipients of the T cell depleted marrow allograft. They increment to 1,000 and stays there for 8 hours and increments to 3,500. Then the weekend occurs and we wanted to see if he had recovered his own counts. He had not. The same

saw-tooth pattern, the weekend, and then his own counts finally returned.

We looked at the one-hour post transfusion increment in the ANC level as a function of the number of granulocytes transfused for these first three patients and there was a direct correlation, which was highly significant, with the increasing counts as a function of the number of granulocytes transfused. This graph is perhaps less steep because there were only four points.

How did they do overall, though? They clearly responded in terms of their infection in the short-term. The first patient is in complete remission and back to her everyday activities now. Her diverticular phlegmon was removed surgically after her own white count recovered. The second patient died two months after the course of granulocyte transfusions due to multi-organ system failure as a complication of bone marrow transplant. Aspergillus was present at autopsy but was not thought to be contributory to his death. The third patient stabilized, eventually was discharged from the hospital and is still alive now with chronic graft versus host disease. Serial one-month skin biopsies were obtained and at day 30 and 60, he still had fusarium in his skin, but at day 100, the

fusarium was gone. So it takes a long time to clear invasive filamentous fungus infection.

Here is our summary of the 14 total neutropenic recipients of G-CSF mobilized granulocyte transfusions to date at the NIH Clinical Center. You heard about the 19 patients at the Fred Hutch, and the data are very, very similar. These 14 individuals received a total of 135 granulocytes that had been mobilized with G-CSF plus dexamethasone. In some cases I have the total number of G-CSF mobilized granulocyte transfusions in parenthesis because the total reflects both dexa and G-CSF mobilized products. We have given this product to 4 patients with aplastic anemia. Three of them had invasive fungus infections, Aspergillus or fusarium, and one had a strep pneumonia. All four patients initially improved. These two stabilized to an impressive degree. The fusarium resolved completely. That was the previous patient. And the strep infection or the pneumonia promptly improved. However, in the first two patients, eventually since they didn't recover their own counts, they had ANCs of close to zero and the disease progressed. In our experience here, we give immunomodulatory therapy with ATG and cyclosporin or cyclophosphamide. It takes 6 weeks to see an

increase in the ANC with about 65 to 75 percent of all patients responding, and 6 weeks of an ANC of zero, even in the presence of granulocyte transfusions, is very problematic. So only one was discharged from the hospital.

In the allo-peripheral blood stem cell transplant patients, there were four, three had disseminated fungal infections and one had an RSV pneumonia, which we didn't know at the time. We thought he had a fungal infection or a fungal pneumonia. These two patients -- this patient was started when he was nearly in extremis. The Aspergillus was progressive and he rapidly died. This patient continued to do poorly despite granulocyte transfusions until we realized he had RSV as his main process and not fungus. These two patients improved. I discussed them on an earlier slide. One died of multi-organ system failure unrelated to fusarium. One is doing well. This patient with a non-Hodgkin's lymphoma and two patients with NHL had a vancomycin resistent enterococcus and multi-organ system failure and was an extremis when we started granulocyte transfusions, had progressive disease and died. Another young girl with pulmonary Aspergillus in the setting of HIV infection and non-Hodgkin's lymphoma

had a very nice response to 10 granulocyte transfusions. And we have two patients with LGL lymphoproliferative disorder, both of whose infections, one bacterial and one candida, resolved. And a breast cancer patient with a pseudomonas ulcer of a myocutaneous reconstruction flap within two days worth of transfusions, her own count had returned and she improved. I am not sure we can say much about this one patient in blast crisis at CML who had a presumed fungal pneumonia who also improved, although he eventually died of other complications.

So there were 9 of 14 patients with invasive fungal infections. Overall, 11 of 14 improved and it was not surprising that these two patients in extremis and the one patient with RSV pneumonia did not improve. But the overall hospital discharge was slightly less than half as you just saw from the Seattle experience, and that has been the MD Anderson experience as well.

These are essentially 14 anecdotes. And what we have heard this morning for what we really need is a randomized prospective trial so that they become more than just anecdotes.

We have also in this institution treated a number of patients with chronic granulomatous

disease of childhood, an inherited disorder where granulocytes cannot make phagocyte oxidase and membrane bound oxidase, and they can't handle various types of fungal and bacterial infections and are subject to recurrent life-threatening infections with organisms like Serratia, Pseudomonas, Candida, Aspergillus, and nocardia. We have treated a total of 10 such patients with 220 transfusions in the past two years. Again, this is a set of 10 anecdotes. And in the absence of a prospective trial, there is not that much that can be said except that 9 out of the 10 had resolution of infection and 9 out of the 10 were discharged from the hospital. And whenever you see such an excellent response, it makes you think that perhaps the granulocytes were involved in this excellent response. And what makes me have some confidence in saying that was that in one patient here and one patient here, they only received two granulocyte transfusions before an anamnestic response in their HLA alloantibodies became clear and they had very significant pulmonary transfusion reactions. We stopped the transfusions after two and three transfusions, but they still resolved their fungal pneumonias and their bacterial pneumonias with excellent antimicrobial therapy. So again, in the

absence of a trial, it is hard to say exactly what the role of granulocytes, even G-CSF mobilized granulocytes, is in patients with CGD, although there is some controversy among the clinical care staff, Dr. Malik and colleagues here at the Clinical Center, about the role of granulocytes.

This is all 14 of our neutropenic patients. These are the white cell increments. This is the incrementing count, not the absolute count in that patient -- but the increment following transfusions of G-CSF mobilized granulocytes. On the x axis are hours after transfusion, and please notice this is not a linear scale. There is pre-transfusion, 1 hour, 4 hours, 8 hours, 24 hours, and 30 hours. And there aren't points at each of these time intervals. There is always a point at 1 hour, 8 hours, and 24, but not always at 4. The orange line is 10 patients who did not have either HLA alloimmunization or splenomegaly. The 1 hour post increment was 1,900, very close to the 2,600 that you heard this morning from the Seattle group. And 4 hours later in most patients, that count was slightly higher. It was 2,000. Suggesting that one-hour post-transfusion, there may be some sequestration in organs such as the spleen and the peak increment is not seen until 4 hours. There is

still an increment at 8 hours, a substantial increment over the pre-transfusion count. That persists at 24 hours and still persists at 30 hours to a very low level. If you try and calculate an in vivo or biologic half life, half of 2,000, you get about 20 hours, which is exactly what Dr. Dale reported from his study of radio-labeled cells, autologous cells, in study participants.

I separated out the three patients with splenomegaly, two with non-Hodgkin's lymphoma and one with CML because they had a markedly different response. The 1 hour post increment was in the 300 range, went down to the high 200 range, and then slowly decreased and then went down to 0 at 30 hours. And then there was one patient who we did not know had HLA allosensitization until the first transfusion was given. He also did not respond as did the mean group without HLA allosensitization. But interestingly, he had some increment. It wasn't 0. That is an n of 1, so I can't say too much about the amount of increment you can expect in an allosensitized recipient. He had a multi-specific positive lymphocytotoxicity screen. So this was a very impressive alloimmunization in vitro. This individual was intubated and had monitoring of every possible pulmonary parameter in the ICU, and we

looked carefully at whether there was 0_2 desaturation, increased need for positive index pressure, decreased compliance of the lung, increased temperature, chills, et cetera, related to the transfusion of HLA incompatible very large numbers of granulocytes and we did not see it in this n of 1.

There was one patient who received a total of 11 granulocyte transfusions early in the course of our work with G-CSF mobilized products, and on only five of those occasions were we able to find a donor enrolled in the protocol that could give G-CSF mobilized product. On the other six occasions, she received a dexa mobilized product from donors already participating in our apheresis program that were used to taking dexamethasone. So I could compare in one study subject the response to G-CSF dexa stimulated product versus dexa alone, and there is a marked and significant difference as you would expect. This happened to be the patient that had the highest increment of all of our patients to granulocyte transfusions. This is the increment, not the absolute count. It was 6,500 one hour post-transfusion, rose to 8,400 four hours post-transfusion, and dropped to a high 2,000 level 8 hours later and at 24 hours was still significantly

above her baseline of almost zero as opposed to the response to dexamethasone, where it was a little bit above 1,000 at one hour, and you can see the trend down here.

Alloimmunization, as has been stated, is a major risk of granulocyte transfusions. In previous publications, the instance of transfusion reactions is 90 percent in individuals getting granulocytes who have preexisting alloimmunization and 11 percent of those who do not. In very elegant studies done almost 20 years ago by Jan Dutcher and Charlie Schiffer where they radio-labeled allogeneic white cells with indium 111 and transfused them, they saw increased pulmonary retention of cells and decreased trafficking to sites of infection in individuals who had preexisting HLA alloantibodies. 20 of 20 successfully migrated to sites of infection without HLA alloimmunization versus 3 of 14.

We looked at the NIH retrospectively at CGD recipients of multiple courses of granulocyte transfusion therapy in the era where we used dexamethasone alone mobilized products. And of 18 patients that we looked at, 14 of 18 had developed HLA alloantibodies during the course of these transfusions. So in some populations, and I suspect that would be CGD and also aplastics, the instance

of alloimmunization is extremely high, on the order of 80 percent. Whereas in bone marrow transplant recipients, it is probably much less because of the state of suppression of their immune system.

We looked more carefully at patients with CDG that we transfused. This is the first six that received G-CSF mobilized product. One with Aspergillus bacterial pneumonia, diffuse nocardia, an unspecified fungal pneumonia, bacterial pneumonia, and staph hepatic abscesses. Our protocol said we would not give these cells to patients with preexistent HLA alloimmunization, but the clinicians taking care of these patients were so impressed with the response to granulocyte transfusions in patients without HLA alloantibodies that they prevailed upon us to make a deviation to our standard operating procedure. So in three of these patients, there were preexistent HLA alloantibodies and in one there was prior alloimmunization, but it was not initially evident on the screen although it became evident later. Interestingly, not all patients with alloimmunization had pulmonary adverse reactions. This could be pulmonary infiltrates, fever, chills, dyspnea, or 0_2 desaturation. None of the patients without alloantibodies had reactions. Three of the

patients with alloantibodies did and one did not. 0_2 desaturation was seen in two of the four with allosensitization and was not seen in the other two. So it wasn't uniform here either. An indium labeled allogeneic white cell trafficking scan was done in the individual with diffuse nocardia. She had disseminated skin lesions which should have been easy to see on the scan and all she had was pulmonary retention with no traffic. So in this patient with alloimmunization, again the cells did not go to sites of infection.

Very nicely with the help of Dr. Fleischer and Dr. Malik here at the NIH, a flow cytometric study using a DHR dihydrorodamine stain was done. Dihydrorodamine fluoresces inside neutrophils when the cells undergo a respiratory burst, which is detected by the flow cytometer. Such patients with CGD don't have granulocytes that can undergo respiratory bursts and the DHR is zero percent of cells in the wild type state. Following transfusion, the percent of cells that are phagocyte oxidase positive rises to anywhere from 6 to 64 percent, starts at 0, and was always greater than 1 percent in all individuals who did not have HLA alloimmunization. It was less than 1 percent -- substantially less -- and this may be some

background activity or background noise, in patients who did have alloimmunization. This high of 54 percent was the first transfusion given to this individual before the HLA alloantibodies became evident in this serum. This number actually decreased to less than 1 percent after the alloimmunization occurred.

I would like to end, as Liana has asked us to, with considerations for the focus of future research studies. I have decided these foci into donor collection methods, components, and recipient considerations. As referred to numerous times both yesterday and today, we need continued long-term follow-up of healthy recipients of G-CSF to see if there are any long-term complications. There do not appear to be with over 1,000 or several thousand individuals receiving not only a single dose but five consecutive daily doses of G-CSF. The other consideration is how often we can do this in a volunteer donor for granulocytes and not for stem cells. FDA allows us to do cytopheresis 24 times a year in a normal donor. Our policy at the NIH transfusion medicine department is to allow donors on this G-CSF mobilized donation protocol to donate no more often than monthly, but that is an arbitrary restriction. Should there be a difference -- should

there be a different restriction for healthy donors getting G-CSF mobilized products than for routine cytopheresis donors?

Collection methods -- certainly with the CS-3000, enhancements and modifications are needed to the apheresis devices to increase the efficiency of collection and those studies are in progress. Components, as we just heard from Dr. Ambruso, it is very difficult if not impossible for most centers to collect, test, and transfuse on the same day or even to collect, test, and transfuse within 24 hours. So evaluation of storage solutions and conditions if ongoing in several institutions is critical to allow this kind of component to be made available at multiple blood centers.

It appears that we have defined a component. The component definition is becoming very crisp. What we found in a retrospective analysis was that 75 percent of our components contain greater than 5 times 10^{10} granulocytes and 90 percent contain greater than 4 times 10^{10} granulocytes in an analysis of about 200 G+ dexa mobilized components. So you can define a minimum number. You can define the apheresis procedures and the donor preparative regimens to yield that product. So the product definition again is not so

much a problem. What is a problem is determining efficacy.

So studies of recipients -- and we have heard this before from other individuals -- a multi-center, randomized, prospectively controlled study, and I think that this will be so expensive and complex that there probably won't be more than one such study which is being organized out of the infectious disease department at the Fred Hutch, or at least there are discussions of it right now, to identify patients most likely to obtain survival benefit.

Eligibility criteria, as we have heard, are deep-seated filamentous fungal infections in the hematopoietic transplant setting, perhaps in patients with severe aplastic anemia, and also life-threatening bacterial infections where we assess the patient as having a greater than 70 or 80 or 90 percent chance of mortality with best available antimicrobial therapy, and in a separate population of patients who are not neutropenic, CDG patients with fungal or bacterial infections.

In terms of assessment of efficacy, we have designed such a trial for patients with aplastic anemia in this institution, which is a referral center for SAA. Our statistician reviewing

our trial told us that our primary outcome should not be resolution of infection but should be survival at three months when she looked at the data we had on patient outcomes in the first 14 patients treated, and that is different than what we heard this morning from Seattle where resolution of infection was a primary outcome. You don't judge platelet transfusions or red cell transfusions by whether the patient leaves the hospital. So this would be sort of a new paradigm. And I am not sure which one of these is best, and I think the statisticians, whether it is hospital discharge or long term survival at three and six months or resolution of infection -- the statisticians should have more discussion on what is the appropriate primary endpoint. The other endpoints can be evaluated in a logistic regression. But you have to define the primary endpoint more clearly.

I would like to stop there with the exception of my last and of course the most important slide, my acknowledgements to Mr. Jaime Oblitas, Virginia Morgan, and Sandy Bangham and the outstanding staff of the NIH Apheresis Center for allowing these studies to take place.

CHAIRPERSON HARVATH: Would the speakers from this morning's two sessions please assemble and

we will give the audience time to put questions together.

DR. ADKINS: Adkins from St. Louis. As a patient, if I had a less than 10 or 20 percent chance of surviving from a progressive infection while neutropenic, I guess I would have a problem being asked to be randomized to receive or not receive a granulocyte transfusion in a study such as this. So I guess I would ask the response of the speakers how they would feel as a physician who is trying to counsel patients for a proposed randomized trial of therapeutic granulocyte transfusions. If they feel that is appropriate or how they would go about trying to convince people to participate on a trial like this.

DR. LEITMAN: That is certainly one of the most difficult questions. Our aplastic anemia trial is not yet running. We have had in the last two months several patients with deep-seated filamentous fungal infections who we knew the mortality would be 100 percent with conventional therapy. The attendings that month were asked whether they would have randomized those patients if the trial were active and they said, oh, I don't think so. But then when we look at our outcome data, all such patients have died with granulocyte

transfusions, 3 out of 3. Only the bacterial infection patient survived. All three had improvement in their infections over the first two to three weeks. But as one waits that six weeks or eight or twelve to recovery of their own counts, it becomes increasingly difficult to support with granulocyte transfusions and then alloimmunization is likely to occur as well. So given the data, though small numbers of patients here and at other sites, I think that that is the way to do the study, to randomize.

DR. ADKINS: I think each of you shared your enthusiasm for this area and actually have been very positive about the outcomes you have observed. I am all for randomized trials to try to prove whether or not these things work. I guess I am questioning is this the right setting as a therapeutic maneuver. In my own talk I will discuss kind of a strategy we have taken as kind of a prophylactic maneuver. I think that that is another way to look at efficacy analyses with granulocyte transfusions, and perhaps a more acceptable way from a patient perspective. So, again, if all of us, let's say, were going to join up and do a Phase III trial, I think it is very important that we are all convinced that we can comfortably go to a patient

and say these are your two options. I don't know which one of these provides the best benefit and I am very comfortable in recommending you to receive or not receive by flip of the coin a granulocyte transfusion. So I just would encourage us all to think about this as we leave today.

DR. PRICE: Well, I might comment. I mean, I agree with you that it is going to be a problem. I personally -- I don't think I would have a problem randomizing. I don't know whether this stuff works or not. But the more we talk about this too, the more there are going to be enough people around who are going to have a problem with that randomization, and I think we are hearing about that now and seeing it. One of the possibilities that we have at least batted around a little bit would be whether in a randomized trial there could be an out and some sort of a deal that says if you are in the control arm and you start going down the tubes that there is an escape clause and you can switch over. So that is another possibility.

One of the problems that we have faced in terms of considering a prophylactic trial is that if you look at the fraction of the patients that get these kinds of infections -- I mean, maybe it is 10 percent of the patients or 5 or 10 percent of the

patients that get one of these kind of infections that we end up treating, that we are going to have to be giving 10 or 20 people granulocyte transfusions for the one we would have given therapeutically. And when you start looking at how much that is going to cost and from a blood center point of view how many granulocyte donors we are going to have to find every day, that is another real problem that comes into it when you are considering the prophylactic.

DR. ADKINS: Maybe I should just stay up here. But I guess, Tom, if this is historically what has happened in the past with granulocyte transfusions, if I am randomized to not receive granulocytes and I have got a fungal infection and it gets worse after that randomization, the tendency is going to be to take care of that patient and to then go on to later give them those granulocytes "off study". And then if your analysis is to determine survival from a statistical standpoint, you are never going to be able to prove that if you allow people to "be rescued" for want of a better word. So this is a very challenging area to try to prove efficacy if you focus this as a therapeutic maneuver. So, again, I think you really have to think about this very carefully based on the

historical trials that have been done with randomized trials. We know that these are practical problems that we are going to run into, and how are we going to deal with them. How are we going to manage then and how are we going to account for that statistically? That is not going to be an easy -- there is no easy answer to that and there may be no answer to that, which is I think the point I am trying to make.

DR. PRICE: I agree.

DR. DALE: I'd just make a brief comment. In the randomized trials that Ron Strauss reviewed, the survival with transfusions was roughly 11 percent for invasive molds. So historically, there is really not much evidence. You would have to base evidence of treatment benefit on new results like were described today. And they are not certainly clear cut at this point to know the benefits. So I feel like from an ethical standpoint, it is a reasonable thing to do, particularly before we and others encourage the more widespread application of this very expensive and resource consumptive technology.

DR. TORLINI: Hi, Sergio Torlini Inova Fairfax Hospital. Since the granulocytes go to a population that is almost 100 percent immune

suppressed and therefore at risk of graft versus host disease and therefore the products have to be radiated, I would like to know if anybody has any data as far as the radiation on granulocyte function, I mean anything recent, and also on the dose of radiation on that.

DR. DALE: I will comment briefly. We haven't studied that in Seattle. At MD Anderson, there are some recent studies that do suggest some modest degree of cell injury with radiation. That may, in fact, be overcome by the addition of other cytokines, the cytokine being gamma interferon, for example, having a protective effect. It is a murky area, though. And basically I think what has been done universally is to irradiate cells assuming they are not damaged. So it is something that needs to be better studied.

DR. LEITMAN: There is a very nice study by Kasberg, Lur, and colleagues in Switzerland published in Blood in 1993 where I can't remember the dose, it was 2,500 or 3,000 centigrade, were given to the product and before and after radiation very careful studies of migration and chemotaxis and fungicidal and on the respiratory burst activity. There were 5 or 6 parameters looked at and there was no difference pre and post to radiation.

DR. DIAZ: Jose-Luis Diaz from Mitre Pharmaceuticals in La Jolla. I have a simple nuts and bolts question for Dr. Ambruso, which is how did you measure apoptosis in the cells that you were measuring?

DR. AMBRUSO: How did we measure it?

DR. DIAZ: Yes.

DR. AMBRUSO: We looked at morphologic criteria. That slide that I showed you showed a distinct change in the morphologic appearance of the nuclei. And we looked at percent of live apototic cells.

DR. DIAZ: Right. And the other thing was in some of the other functional assays, how did you distinguish the response you were getting -- how did you determine that the response you were getting was from a neutrophil and not from something like a monocyte, for example. Obviously monocytes can burst, et cetera.

DR. AMBRUSO: Sure. Our preparations are 95 to 98 percent neutrophils, and that was checked each time that the cells were separated.

DR. DIAZ: Oh, I see. So you took the samples and then did a separation?

DR. AMBRUSO: Right. There was a further separation.

DR. DIAZ: Okay. Thanks so much.

AUDIENCE MEMBER: I would like to comment on the respiratory distress that you saw, Susan, with some of your granulocyte preparations. We see a lot of respiratory distress after transfusion and only the anti HLA2 agglutinating antibodies of the HLA antibodies apparently cause respiratory distress. Now the antigranulocyte antibodies for the granulocyte antigen specifically also cause respiratory distress, and we find that we have many, many more antigranulocyte antibodies causing respiratory distress than HLAs. But in HLA territory, if you can avoid giving anti-HLA2's -- giving HLA2's to the people who might have anti-HLA2's, you might avoid that small group. But they look to be only about a fifth or a tenth of the respiratory distress cases that we have before us. So I wonder if anybody else has any different experience with that. We have been very interested in that because it is a very serious complication.

DR. LEITMAN: In the retrospective analysis of pulmonary complications in CDG recipients of granulocytes that was published by Dave Stroncek a year ago with the CDG patients here, the pulmonary reactions were seen with HLA alloantibodies alone in the absence of anti-

neutrophil antibodies, and all patients were screened for presence of both anti-neutrophil and anti-HLA. I am almost certain that it wasn't only HLA A2. Most of the recipients had multi-specific antibody screens. I am not sure we can pull out the A2. But I don't think it was only HLA A2.

DR. PRICE: Becky, are you referring to granulocyte things only or is this trolley type stuff you are talking about?

AUDIENCE MEMBER: This is trolley type stuff. Because we have seen it with the anti-granulocyte. We have seen it anti-HLA2, but with other kinds of anti-HLA. I would certainly be willing to learn. I need to go read David's paper again obviously.

DR. AMBRUSO: Pulmonary reactions related to transfusions is something that our laboratory has been interested in in the past few years and it certainly occurs for reasons other than antibodies. And these patients, aside from the granulocyte issue, certainly are receiving other products and have other illness-related problems that certainly could predispose them to that. I am surprised that, in fact, there aren't more and it is something, more reactions, and may not necessarily be related to the antibodies or the granulocytes

themselves. But there are other compounds that have been indicated -- cytokines, lipids that were intensely interested and that certainly have an etiologic role. So this gets very murky when we start talking about pulmonary reactions and there are lots of ways that it can happen.

DR. KLEIN: Harvey Klein, NIH. Is anyone looking at or concerned with the issue of CMV transmission when patients who are immunosupressed get these large numbers of granulocytes over long periods of time?

DR. PRICE: Well, I mean the other people can answer this. Our routine, of course, is for any recipient who is CMV negative to provide donors that are CMV negative. Whether a CMV positive donor getting CMV positive stuff is going to get some other strain and do him some damage is not something that we have addressed.

DR. AMBRUSO: We have routinely used the same or adopted the same approach and that is either using sero negative or using leuko-reduced. But most of what we are doing still is sero negative -- CMP sero negative for patients that would fall into that category. And I assume we would continue.

AUDIENCE MEMBER: Could I ask anybody, what would be the upper level of neutrophils that you would exclude a person from this proposed trial?

DR. DALE: Do you mean what --

AUDIENCE MEMBER: Yes, what degree of neutropenia do you think that you would think neutrophil transfusions would have an impact?

DR. DALE: That is a good question. The traditional or historic level of 500 is a level that is cut off as a pretty high level. The risk of infection is the severity of infection is considerably more if you use 200. You could argue for using even a higher threshold based upon studies of functional deficiencies of the neutrophils produced after transplantation in the early phases of hematopoietic recovery. I don't think that any choice would be less than arbitrary, though. It would fall somewhere probably between 200 and 1,000.

AUDIENCE MEMBER: Could I just ask another question? If you were treating a fungal infection, would you disqualify people for this trial on steroids?

DR. DALE: That is another very good question. In the post-transplant period when people are on steroids, that is going to be an important factor in their infection. And it has been thought

that it would be useful to transfuse people with higher counts because again the endogenous cells are perhaps ineffective. That is mostly speculation, though. I don't think anyone -- there is not a right answer. So that whatever trial were conducted would be some consensus. But certainly people I have talked with have suggested that for fungal infections after transplant for people on steroids, you should consider people with higher counts than 200 or 500.

AUDIENCE MEMBER: But wouldn't they suffer, then, the same paralysis that is going on in vivo?

DR. DALE: Yes and no, and that depends upon whether the effects of steroids or immunosuppressive drugs have their effects on developing cells or developed transfused cells. And I suspect it is the former, not the latter. But, again, another good research question.

DR. STRONCEK: Dave Stroncek, NIH. A couple of comments. One is I know the average data looks very good for the increments and neutrophil counts after transfusions and patient outcomes. But I have seem some of these same patients anecdotally and I don't think the average data -- I don't think the whole picture is quite as rosy as the average

data tends to show. That said, I think when you consider clinical trials, I have a similar concern about the patients studied as Dr. Adkins but for a different reason. I think bacterial infections granulocyte transfusions will work great for. But the number of patients we see for that have bacterial infections not responding to antibiotics is really very rare. The biggest problem seems to be patients with fungal infections. And I have a concern that the granulocyte transfusions may be effective in increasing white count, but they still might not be effective in resolving fungal infections, or at least not effective enough to see in a reasonable trial. So for that reason, if you set up a trial that just looks at treating fungal infections in neutropenic patients, it may fail. So it may be worthwhile to try it in a different patient population, maybe in the prophylactic setting.

And the other comment too is I know in the past we had to look at really patient survival as an outcome because that was the only outcome measure we had. You couldn't measure increase in granulocyte counts. But I think that needs to be relooked at in further studies. A number of the studies on cytokines that have been given to

patients with chronic neutropenia and neutropenic patients getting chemotherapy have shown shorter days of neutropenia and shorter days of hospital stay and decreased febrile incidence, but I don't think they have been held to the same standard to show that there is less infection -- less fungal and bacterial infection. So I just caution that we don't set ourselves up to fail because we are being more strict with the criteria to say granulocyte transfusions don't work. If you do it because we are more strict than any other standard we hold for other blood products or other drugs.

DR. HENDERSON: I am Theresa Henderson from Georgetown University and I have a couple of nuts and bolts questions for Dr. Ambruso. I just wanted to clarify that the cells that you were testing were not the apheresed product but had been stored and cleaned up by fical or something like that?

DR. AMBRUSO: Right. The actual data that I have showed was cells from the -- they were drawn from the patient before any G-CSF had been given. And approximately one hour after the dose of G-CSF on day 4. So that is the fifth dose of G-CSF. Subsequent to that, the patients underwent granulocyte collections and I didn't show the data

but put it in a summary slide that we looked at additional studies in those granulocytes. And we really didn't find much difference in terms of the function for the first 24 hours. I had 24 hours of storage.

DR. HENDERSON: Oh, that is good to know. And you said that you looked at C-11B and saw no significant changes. I wondered if you looked at Selectin-62?

DR. AMBRUSO: We did not.

DR. HENDERSON: And you also touched on the cytosolic calcium increase. I am sorry, I don't understand -- I am new at this, so I don't really understand the significance of looking at that. Could you enlighten me?

DR. AMBRUSO: We did that in part because we wanted to look at a biochemical parameter that we thought was related. I mean initially we did it and were excited related to the chemotaxis. But obviously calcium and signaling related to calcium is something that is significant to all functions of the neutrophil. We also did it in response to some studies in another patient group. There is a group of patients who have neutropenia and neutrophil dysfunction that are associated with glycogenesis 1B. These individuals, when you put

them on G-CSF, their neutrophil counts are restored. They are not neutropenic. They still have a chemotactic defect and their cytosolic response to calcium, which was aberrant to begin with without treatment with G-CSF normalized. So we wanted to look at a group of control individuals given G-CSF to see if that affect was consistent. It is consistent. I don't know if it has any relationship to the defects or anything that was shown in the function of the cells in normal patients given G-CSF.

DR. HENDERSON: Thank you.

DR. DIAZ: Just a quick point. About L-Selectin. We did some studies on isolated neutrophils looking at 62L, which is marked for L-Selectin, and what we found was that in isolating neutrophils, the L-Selectin dies off slowly and after about 24 hours, there is only about 50 percent. And then by about 48 hours, there is only about 20 percent left. If at any of these points you actually activate the cell by giving it a stimulus, it disappears within an hour or very quickly.

DR. LEITMAN: I have a question for the FDA. You can now define a product much better than you could before. You can now define a measurable

outcome in a transfusion recipient. You can even set criteria for assessing that outcome, an increment in the granulocyte count at certain times that is sustained for a certain length of time. So your definition of the component of how you treat the donor and what you measure immediately after transfusion in the recipient is quite well defined. Would the FDA, in considering licensure of this product, have to see, want to see, insist on seeing the results of a randomized clinical trial? And what would be the endpoints that they would look at of that trial?

CHAIRPERSON HARVATH: Susan always asks me these tough questions. That is why we are holding the conference. We wanted to hear from you what you feel is an appropriate approach because we have heard that this is very expensive. We also hear that normal donors may be asked to be receiving cytokine repetitively. And as you know, we have to constantly look at risk benefit in terms of public health issues. I agree with everything you have said. I think that you have made enormous strides in defining a component and in terms of the cell biology. I think that that is the easier part of all of this. One of the things we wanted to hear from this workshop and yesterday were your concerns

about the exposure of normals and the effects long term. So I don't really have an answer for your question, Susan. I think what we wanted to do was hear what you were all comfortable doing. We know some folks have talked about collectively doing a randomized trial. We know the NIH is interested in hearing what the interest is in that and in determining whether there would be support to try and do that. We also know that is going to be very expensive to be done and I think you said it very accurately this morning that it probably would be done once and hopefully done so that one gets a clearer answer.

So I honestly don't have an answer. I can't speak on behalf of the Agency. As you know, I am interested in the cell biology and also I think we have an obligation to ask investigators who are collecting these products how comfortable they are with giving their normal pedigree blood donors cytokines and giving them cytokines perhaps on more than one occasion.

One question I have for all of you along that line -- and I am sorry I don't answer your question but that is my non-answer. One question I have for you is I have heard all of you say that you are working with your pedigree donors. So you

really have a very clear picture of their medical histories and their hematologic picture. When you do a complete blood count on someone, are you including a differential in that and how would you feel about giving G-CSF or some other cytokine to someone where you only had a blood count and not necessarily were resting assured that they had a normal differential? Do any of you have concerns that there might be an individual perhaps with perhaps a pre-leukemic state that could possibly be missed? That might not be your normal pedigree donor population that you have followed over the years and have a clear health picture on. But something we have kind of wondered about and we don't have the answer to it is what kind of -- would you include a differential in your workup initially in a new donor situation? And that is for everyone.

DR. PRICE: Well, we do a differential, but by the time we get the answer back, the G has already been given to the donor. Like you say, these are donors we know. But a guy who is going to develop leukemia will do it at point X and that may happen. I think -- I mean, my read on this is that there have been a lot of people looking at this and the evidence of giving one shot of G even to

somebody who is developing leukemia that it is going to do him any harm would be about zero.

DR. AMBRUSO: We, like you, are doing counts with automated differentials on our granulocyte donors as well as we are doing counts on platelet donors. That is something that we worry about and I wonder if you would be more concerned or more likely to run into problems with very high counts -- just with the white count and looking at that carefully than with the differential. I am not sure that we collect a lot of reasonable data. The parameter that should be different is looking at counts that exceed or are above or below certain levels in terms of the risk for leukemia or other problems.

DR. LEITMAN: We automatically do a CBC with an automated differential on all our donors including platelet donors, and part of the reason is to look at their platelet counts to qualify them for the next platelet pheresis donation. Because the granulocyte donor one month later is most likely to be donating platelets and not granulocytes again. And that is the reason I think most centers if not all have to do a pre-pheresis count. Like Tom, our count is obtained the day of pheresis in the current iteration of the study so that we get the count

after they have been given the stimulant. Does the count have to include a differential? It is almost a moot point since the automated instruments provide the differential. But I don't see in the absence of a trial the critical necessity for a differential.

DR. DALE: I would just comment too that this safety issue, that is, a single dose must be an extremely small risk. Because the substance that is used as a drug is really very close to the natural hormone and a surge of this magnitude probably happens many times in a person's lifetime.

AUDIENCE MEMBER: I have a question. What are the legalities about giving G-CSF to an unrelated donor? I know that in our institution we would have to go through IRB to get this approved. Is that pretty much the way it stands for everybody right now?

DR. DALE: We have -- we do it with IRB approval and informed consent.

DR. LEITMAN: I think in this institution, all G-CSF given to normal donors on numerous protocols, not only ours, is done with IRB informed consent.

AUDIENCE MEMBER: All right. And I guess a follow-up question is what exactly -- how do you modify your informed consent form for a G-CSF?

I mean, how extensive is it? Several pages? Just a paragraph?

DR. LEITMAN: It is four pages.

AUDIENCE MEMBER: A paragraph. Thank you. We were thinking of producing a movie to go with it to show.

DR. PRICE: And I think that although maybe everybody up here does it in IRB, I think there are places around who don't do it in IRB. I still think they have a consent form and explain to the donor what is going on. But I figure that there is enough information around that as long as they are keeping track of things that it is not really a research thing. I think that is not undefensible. Did I say that right? Too many negatives?

AUDIENCE MEMBER: Having an almost 20-year history in apheresis collections and a nursing background, I have to preface this by saying that I am a donor advocate and having been a nurse and being trained to try and help people get better, I have a real internal conflict going on with asking volunteer donors to take a drug that we don't have long-term studies as to show what happens with these donors with exposure to the drug. And then tied in with that is the fact that there really -- if I am hearing correctly what I have heard today, the

efficacy of what we are trying to collect really has not been established. And I am real worried and terribly protective of the donors and what we ask of them. I think also as a personal observation of donors that we have worked with in the past, yes, the donors will come back after many circumstances and I would be willing to wager that because they are a captive audience, they are a very dedicated conscientious group of people that would probably -- and I acknowledge that I have no data to support this fact other than a gut feeling -- but I think the donors would probably come back no matter what we asked of them, and where do we draw the line between what we ask of our donors and what we are trying to accomplish in our patients?

DR. DALE: I would just comment. I think that is a very good statement and in fact provides part of the rationale for a randomized trial. It is not to do something with donors which is not convincingly proven to be of value. Protect their interest as well. On the other hand, I think being open with people and honest about the potential benefit and the known risk that the donor population can make an informed choice. As adults, some will say yes and some will say no and that is okay.

DR. AMBRUSO: I think you bring up an important point and I think we really do need to know long term effects. I guess I am not concerned about what happens with one dose of G-CSF and then the donor goes merrily on their way. People who are involved in providing blood products know that you are relying on the bone marrows of a very few individuals to support the rest of the community. And it is really not a single donation per year that we are concerned about. It is the donation of something of someone who might undergo this procedure many times in a year and that is completely undefined. As you were saying this, I am reminded also that we have many, many dedicated platelet donors. And you know, we don't know long-term what happens to somebody who is having their platelets collected many, many times a year -- 23 or 26 times a year. You know, we don't have long term data on that, but we still go ahead and do those times of collection. So this is a muddy area, but I think we really do -- with G-CSF mobilization, we do need to get some long-term data, particularly on the donors who are going to be dedicated and are going to donate more than once a year. If they are going to be denoting once a month, I think there is some long term information that we need on them.

DR. LEITMAN: The NIH has been collecting granulocytes for transfusion for greater than 15 years, maybe for 20 years. Since the Michler studies in the 1970's about starch and steroid to optimize collection efficiency, donors have routinely been given dexamethasone plus starch. Neither of those medications are licensed for use in obtaining granulocytes because granulocytes aren't a licensed product. So the past two decades in this field has been characterized by giving donors drugs that do have adverse effects. 45 percent of donors getting dexa don't have a good night sleep. They have nightmares and insomnia. I see nothing in the past decade of a history of administering G-CSF to normal, healthy individuals that increases my concern that G-CSF has any long term, adverse consequences above and beyond what we know about dexamethasone and starch. Acutely, certainly, it causes more discomfort, which is why I think you can expect a 10 percent dropout in individuals participating in this, which is fine.

AUDIENCE MEMBER: I would hope -- this may be rather unpopular, but I would hope that the FDA would like to see or would want a randomized trial, an appropriate trial. I think one could ask the question that if you cannot demonstrate

efficacy, number one does it matter and number two, who do you decide to give it to? Are you going to give it to a huge number of patients who may not need it or who don't need it or where you can't demonstrate efficacy? So I think to study it and to determine how you ought to use it, you really need an appropriately designed trial. It may be too late to do that and this is a plea, perhaps, fir people to begin to do appropriately designed trials very early in the course of the development of a new component. Many years ago -- some of us are old enough to be more interested in history and perhaps unrecorded history.

Everybody knew that fibrinogen given to a woman bleeding from low fibrinogen after a pregnancy that the fibrinogen was effective until a major university did a comparison trial of fibrinogen versus no fibrinogen and found that the increase in fibrinogen was as rapid without it as it was with it. That was a pretty well defined derivative. It could be measured and you gave a dose that you knew about. So I think being able to define a component does not necessarily mean that it is going to be effective and useful.

DR. SHAPRIO: Arell Shapiro from Life Source. From the data presented, I didn't get a

very good understanding of how the patients are treated. Is it -- you know, once it is ordered, is it daily? I mean, Dr. Leitman, in your presentation it was daily except for the weekends. Is that how -- it is just ongoing until the person either recovers back their white count? I mean, what are the endpoints and when do people give up?

DR. LEITMAN: The call comes to the consult service to consider granulocyte transfusions for a patient. One of our fellows immediately within hours sees the patient and performs a full evaluation and it is not a 100 percent approval. The Transfusion Medicine Department takes a very active role in deciding and looking at everything involved with that patient as to whether this is a good thing to do. Once we start, it is daily, omitting in general weekends but depends on how critical the patient's status is and we do have the potential to collect on Saturday and Sunday and holidays, which we do.

You raise a very good point. In some individuals, the increment after the first two transfusions is so high and so sustained one can ask whether it is necessary to continue daily transfusions especially in small kilo recipients such as children or small sized adults, and in such

individuals on such cases with discussion with the clinical staff we have moved to every other day transfusions and the neutrophil count does not diminish generally to zero.

AUDIENCE MEMBER: So basically you just do it by following the patient and see what their response is. Have you thought about doubling up on a Friday so that you could sustain them over the weekend -- give them a double dose?

DR. LEITMAN: The data on storage for longer than 24 hours is problematic. So we do it every day.

AUDIENCE MEMBER: No, I mean infuse both on Friday.

DR. LEITMAN: Since the increment is in the 2,000 range with the single transfusion, that is enough for us. We have not done double doses.

DR. PRICE: Our general approach is similar. Once we start, we try to do it daily. We even do it on the weekends. The endpoints are a little bit muddier. Part of this comes back to the question that was asked earlier of what is a good neutrophil count to have.

And once you get it up to 2,000 are you okay or is somebody with a fungal infection, would they rather have a count of 8,000 than they would of

3,000? And our feasibility study didn't really control that and it was kind of up to the clinicians. But that would have to be very carefully laid out for a real trial.

AUDIENCE MEMBER: We have donors who are willing to take G-CSF for many days in a row. Have you looked at seeing if you can collect granulocytes from your community pools on a daily basis for say four or five days and would this be of any benefit?

DR. PRICE: We haven't. Part of this is that I -- I mean, I think you are in two different ball games when you are talking about a one-shot thing and a multi-thing as Dan was talking about. I mean, you run into are the cells the same after five days, say of G, as they are 12 hours later. You also run into issues of cell separation efficiencies if the cells aren't really different.

But I think at least from my point of view, that wasn't something I was willing to ask a regular pheresis donor to do. We have -- as you know, Scott, on our early studies we did that with the bone marrow donors from the Hutch. And when we did that, there were several of those donors that did have some problems getting daily starch.

We had to stop collections because people got fluid overload and got bad headaches and

things like this. It didn't happen all the time, but there was enough of that that for our community donors it seemed to me that it was sort of above and beyond the call of duty just to do it once.

AUDIENCE MEMBER: That is the reason why we use pentastarch in our repheresis unit because of the multiple collections that they will be undergoing over -- up to 12 days is what we allow at our center.

DR. LEITMAN: I just want to agree with Tom Price. There is a limit beyond which I will not ask a normal healthy community volunteer to do something even under the auspices of trial, and I think one granulocyte aphoresis collection is all I would ask them to do rather than serial. We have occasionally, when we have been unable to get a donor in over the holidays or whatever, I have had a donor donate two weeks after their last donation. But that is a very rare event, once a month is what I feel comfortable asking an individual to do. It is really an entire day dedicated to apheresis. They don't feel that well afterwards. Their work is affected and their home life is affected. I think that is as much as I would ask.

DR. PRICE: And another issue that comes up there, and I don't know if you are still doing

this, Scott -- but when we were doing the family donors, those guys had central lines and that is also something I am not too interested in having a regular community donor have to have.

DR. CONFER: Dennis Confer, National Marrow Donor Program. On the issue of G-CSF safety, I hope it is sort of a rhetorical question, but the question is, is there really anybody among us who would give G-CSF to a normal donor if in fact we thought there was a reasonable chance that it would cause some late hematologic effect? If we thought that there was some reasonable chance that in fact these donors would develop leukemia at a higher rate or if we thought that there was some reasonable chance that in fact these donors might develop aplastic anemia at old age, I think none of us would give G-CSF or any other hematopoietic growth factor to a normal donor. I think in the same time, I am convinced in making preparations to give G-CSF to volunteer hematopoietic stem cell donors, I am confident that this drug has no long-term effects. But I think to demonstrate that, the best way is to collect the long-term follow-up data.

And we will make plans to follow these donors for as long as we can. And it is interesting because it is something that we talked about

yesterday, that we haven't done it with bone marrow donors. That is truly a deficiency. We also need to follow the routine bone marrow donors for as long as we can to prove that that in fact doesn't cause late effects, which again we are confident or we feel reasonably confident that it doesn't.

But the other thing to keep in mind is that I can assure you that people who get G-CSF will develop leukemia. Because normal people develop leukemia who have never been exposed to G-CSF. And the real question is not how many cases or whether people develop leukemia, the cases is how many cases develop and is that different than what would have been expected among a normal control population. So the data we collect really has to be very comprehensive.

And it has to be compared to an appropriate control population in order to determine whether the incidence of leukemia is, in fact, excessive. Because it will occur. And I know from experience that it has occurred in bone marrow donors, both before and following bone marrow donation. So it is a matter of how much, and that is just going to take a long time to answer that question.

DR. PRICE: Yes, I think -- because what we are doing is the mortality of our community donors eventually is 100 percent.

CHAIRPERSON HARVATH: With that concluding statement, I would like to thank all of you very much for I think a very informative session this morning. We will convene in an hour.

Thank you.

(Whereupon, at 12:05 p.m. the workshop recessed for lunch to reconvene this same day at 1:10 p.m.)

1

2

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

(1:10 p.m.)

DR. STRONCEK: I am Dave Stroncek and I am going to moderate this session. We have three presentations and then after that we will have a panel discussion. Following the panel discussion, we only have three abstracts which will be presented and then a little further discussion. So we anticipate we will probably get done a little early today. But the first lecture today will be by Dr. Douglas Adkins. Dr. Adkins is Assistant Professor of Medicine at Washington University School of Medicine, and he is the medical director of their National Marrow Donor Transplant Collection Program. And he will talk to us today about granulocyte product evaluation.

DR. ADKINS: Okay. I'd like to thank Liana Harvath for inviting me to discuss our data from St. Louis on granulocyte transfusions that are mobilized with G-CSF. Could I have the first slide? Liana has asked me to discuss product evaluation in this area, and I will focus on cell dose and leukocyte compatibility. Because at least at this point in time in my opinion, these are perhaps the two most important issues in this area today.

I don't have any specific recommendations about either of these two issues, but I would suggest or put a vote in to provide additional research support to determine efficacy parameters as they relate to product evaluation.

In my opinion, granulocyte transfusions may be the only strategy which has the ability to eliminate severe neutropenia after high dose therapy, which is obviously the reason to pursue this area. It is important to ask what are reasonable goals to achieve with granulocyte transfusions. Certainly it would be nice to show that these products reduce febrile days, antibiotic requirements, reduce the occurrence of documented infections, and to successfully treat documented infections as we have discussed. Perhaps in high risk populations, one might be able to demonstrate reduced mortality.

I think as you have already seen this morning, it can be very difficult to demonstrate these efficacy parameters in clinical trials. There are many causes, for instance, of febrile days beyond just an infection. Transfusions can cause that, antibiotics, et cetera. So it introduces a lot of confounding variables in trying to set up a well-designed clinical trial in this area.

Why use granulocyte transfusions to prevent neutropenic infection related complications? Well, certainly there are preclinical models, as you know, that demonstrate a clinical benefit in terms of improve survival with granulocyte transfusions. In addition, there are two meta-analyses of randomized human trials that also demonstrated a clinical benefit, but only if certain conditions were met. This is data from two decades ago published by Fred Applebaum demonstrating the critical importance of component cell dose on efficacy. In this case, which is a model of neutropenic dogs with Pseudomonas bacteremia, this data demonstrated the steep dose response curve that they observed in this trial. And if one was able to infuse products containing more than 2 times 10^8 per kilogram, uniform survival was observed. And interestingly, the same threshold dose would correlate with significant increments in ANC. This kind of data suggests to me that it is important to demonstrate significant increments in the ANC to go on to demonstrate measures of efficacy. And interestingly, in a 7 kilogram human, this threshold cell dose correlates with about 1.4 times 10^{10} granulocytes.

As I mentioned, two meta-analyses of randomized trials of granulocyte transfusions in humans have demonstrated benefit. In the analyses of prophylactic granulocyte transfusions, granulocyte transfusions that contain an adequate dose of leukocyte compatible components resulted in decreased relative risk of infection, mortality, and death from infection. In the analysis of therapeutic granulocyte transfusions, these products resulted in improved survival if an adequate dose was administered and if they were given to patients with inherently low risk or low likelihood of survival.

The combination of these trials suggest that cell dose is a very important determinant of efficacy and perhaps leukocyte compatibility as well. These are trials of non-G-CSF mobilized granulocyte transfusions.

Historically, limitations of granulocyte transfusions have primarily been issues revolving around low cell dose and the presence of leukocyte incompatibility. Both of these factors are probably the primary determinants or the primary cause of poor and nonsustained increments in the ANC with granulocyte transfusions not mobilized with G-CSF. Another important problem in prior trials was the

frequent occurrence of febrile transfusion reactions, a problem which confounded the analysis of efficacy. It is my hypothesis and perhaps that of others in this room that significant and sustained post-transfusion increments in the ANC may be a key pre-condition for demonstrating reproducible improvements in clinical outcomes with granulocyte transfusions. So that became the focus initially of our research in this area.

Let's look at ways of improving component yield or component cell dose. Most people in the last few years have been focusing on trying to increase donor ANC, and certainly as Tom Price has shown, that is probably the most important determinant of granulocyte component yield. And so with growth factors such as G-CSF, we can clearly increase the donor ANC pre-collection. There are apheresis factors, though, as Susan Leitman has eluded to which we need to work on to try to improve component yields with apheresis. I will talk about our data with varying the interface offset and showing how that influences component collection efficiency and yield.

This is a table that I put together which shows you the component granulocyte dose based on choice of mobilizing agent. And as has been

discussed earlier, with granulocytes collected at steady state and without a red cell sedimenting agent, one can only collect about .1 to .3 times 10^{10} cells. With corticosteroids and hetastarch, the average collection contains about 2.3 times 10^{10} cells but no better than 3 in general. With G-CSF, it has been our observation that one can increase component cell doses by a factor of several fold, number one, and number two, the larger the dose of G-CSF that we have given to normal donors, the greater the component yields, as you can see here. We have already seen data from Seattle and also from this institution showing that there is an added benefit of giving Decadron along with G-CSF and resulting component yields. I am aware of at least one study that has used GM-CSF to collect granulocytes, but that was an abstracted report which did not comment on component cell doses.

As I mentioned, although most people in the last few years have focused on ways of increasing donor white count as a strategy to increase component yields, we have also looked at the effect of altering apheresis parameters. One that we have looked at is the interface offset setting. As Susan mentioned, the machine -- using the Baxter device, the machines default setting is

15. And to my knowledge, there are actually quite a few centers that do use that particular setting, even today. So we did this study where we collected granulocytes using three different interface offset settings varying from 15 to 25 to 35, and we determined the effect on granulocyte collection efficiency and granulocyte yield. These were all 7 liter pheresis and donors receiving G-CSF at 5 mcg per kilogram along with hetastarch.

So as you can see, as you increase the interface offset setting, you improve granulocyte collection efficiency from 40 to 60 percent. And this results in improved yield as you can see here. So our practice now is to use an IO setting of 35 instead of our old practice of 15.

This is data we recently published demonstrating again, as others have, that significant and sustained increments in the recipient ANC occurs with transfusion of G-CSF mobilized HLA matched granulocyte components. Our initial clinical model was to collect granulocytes from HLA matched sibling bone marrow donors who were receiving G-CSF and transfuse these products as prophylaxis against infection. The advantages of this model was that we chose allogeneic bone marrow transplant patients who had an expected interval of

severe neutropenia during which most indeed developed complications of that problem. There was also an available HLA matched or leukocyte compatible granulocyte donor, and that person would be motivated to undergo frequent granulocyte collections for their sibling. In my opinion, prevention of infection may be an objective more easily reached with granulocyte transfusions than successful treatment of established infections. Many times these are associated with multi-organ failure or confounding causes for infection which complicates the interpretation of efficacy trials.

The limitations of this model is that the donor has to be ABO compatible and the donor must be HLA matched. And obviously the donor would have to agree to undergo additional time commitment to participate and perhaps somewhat greater risk over just bone marrow collection.

The objectives of this study were to carefully document the kinetics of the recipient ANC with each granulocyte transfusion. The donor underwent bone marrow collection on day zero, transplant day zero, and then received G-CSF daily for five days at 5 mcg per kilogram. They then underwent alternating day granulocyte collections on days 1, 3, and 5. The recipient underwent marrow

infusion on day zero and then received G-CSF daily from day plus 1 until neutrophil engraftment. Granulocyte transfusions were given fresh on days 1, 3, and 5, and then we carefully determined ANCs pre and post-transfusion as you can see here.

This is a table demonstrating for you the increments that we observed after transfusion of these granulocyte products on days 1, 3, and 5. You can see here that again these are increments. This is the mean one hour and mean peak increments that we observed, and they are quite substantial with the mean peak increment being up to 11,095 cells per microliter. Interestingly, the peak increment typically occurred about 8 to 12 hours after the granulocyte transfusion, not at one hour as you might intuitively expect.

This table demonstrates for you that the increments were sustained with the time after the transfusion in which the mean ANC was above baseline being at least 25 hours. So we ask the question, where do these granulocytes go once transfused. So we took samples of the granulocyte components collected on day +5. We labeled them with indium and then transfused them into the allogeneic bone marrow transplant patient and we monitored scintographic scans serially. This is a scan

obtained within four hours of granulocyte transfusion demonstrating very intense uptake in the lungs. 24 hours later, you can see that the lung uptake has essentially dissipated, with intense uptake now in the liver and spleen and the marrow. So it looks like these granulocytes initially, once infused, immediately track for the most part to the lungs and then are probably gradually liberated over many hours resulting in that peak ANC that I mentioned to you earlier.

We also wanted to know, based on these kinds of assessments, were these granulocytes functional, that is, do they localize to sites of inflammation after transfusion. Again, this is another indium scan of G-CSF mobilized and HLA matched granulocytes. This is a scan obtained within four hours of infusion demonstrating predominantly lung uptake and spleen. This is a scan obtained 24 hours later. Again, the lung uptake has dissipated quite a bit. But now you begin to see an area of uptake here in the cecum and ascending colon. 48 hours later, this is a very intense area of uptake now, as you can see outlining the ascending colon and cecum. This is a patient who had diarrhea and colitis after their preparative regime. We have similar scans demonstrating this

kind of uptake in the mouth in patients with mucositis. So these kinds of studies along with studies from David Dale and Tom Price's group would support that these are functional granulocytes.

We have just completed a fairly large study of granulocyte transfusions in the allogeneic peripheral blood stem cell setting. The reason we chose to move from bone marrow to peripheral blood was really a practical one. In allogeneic bone marrow transplantation, the duration of neutropenia is substantially longer. And since we are using a single granulocyte donor, who is the stem cell donor, it is very difficult to expect them to do 8 collections over two weeks or three weeks. So from a practical perspective, it was better to pursue the allogeneic peripheral blood stem cell setting where the duration of neutropenia was about a week shorter.

So in this study, we accrued concurrently two cohorts, cohort A and cohort B. Cohort A received prophylactic granulocyte transfusions on days 3 and 6, and these were components that were collected from the stem cell donor who received G-CSF to mobilize these granulocytes. Again, they were obviously HLA matched. Cohort B did not receive granulocyte

transfusions. This was a biologic randomization determined by the availability or not of an ABO compatible, HLA matched related donor. And the donor, as I mentioned, of the stem cells was also the donor of the granulocytes. I have data to share with you on 13 of cohort A. We have 19 actually in cohort A. And 11 of the 51 we have in cohort B. The patients in this study received the same preparative regimen, the same graft versus host disease prophylaxis and the same supportive care, including the way we initiated and stopped antibiotics.

This demonstrates for you that in cohort A that received granulocyte transfusions, we did see significant and sustained increments in the ANC after the granulocyte transfusions given on days 3 and 6, as you can see here.

We then looked at the ANC one day following granulocyte transfusions in cohort A and on the same day in cohort B, and we found that the absolute ANC -- the lowest ANC we observed on that day was significantly higher in cohort A that received granulocyte transfusions. We then tried to determine an efficacy with this preventive therapy, and we have looked at numbers of days of IV antibiotics from day zero until neutrophil

engraftment and have observed that the numbers of days of IV antibiotics in that interval is about 4.5 days less in the cohort that received granulocyte transfusions. One might argue that the proportion of patients in cohort B receiving antibiotics on day zero was greater, and to some extent that was true because we looked at the numbers of days of IV antibiotics in the interval from starting the preparative regimen to day -1, and found that cohort B had received more antibiotics, but only by two days, which in my analysis wouldn't account for this 4.5 day difference we see after day zero.

We do not -- although the absolute numbers of febrile days was greater in cohort B compared to cohort A, this was not statistically significantly different. That may be more difficult to prove in this limited number of patients given the relatively small numbers of febrile days you see here. So to my knowledge, this is the first -- this is preliminary data, but it is the first data that I am aware of that demonstrates a potential clinical benefit of giving G-CSF mobilized HLA matched granulocyte transfusions to such patients.

With that in mind, we were interested in knowing the potential importance of leukocyte

compatibility with G-CSF mobilized granulocytes. I think one could arguably ask, since we are able to collect granulocytes with such a huge cell dose today using growth factors such as G-CSF, can that massive cell dose overcome the problem of leukocyte incompatibility? So that was the question of this trial.

This is a study again with the objective being to determine the kinetics of the recipient ANC after transfusion of prophylactic granulocyte components that are mobilized with G-CSF, but these components may either be leukocyte compatible or incompatible. The donor was a first degree relative of the recipient, received G-CSF daily -- or actually, I am sorry, four doses on transplant days 1, 3, 5, and 7, and the dose of G-CSF we chose in this trial was 10 mcg per kilogram. Granulocyte collections were performed on the evening of day one and the mornings of day 4, 6, and 8. The recipients were all autologous stem cell transplant patients who had reasonably adequate stem cell products as defined here based on CD34 numbers. The recipients received G-CSF daily from day zero until neutrophil engraftment, and then received fresh granulocyte transfusions early morning of day 2 and in the afternoons of day 4, 6, and 8. And then we

carefully documented the kinetics of the ANC of the recipient of these granulocyte transfusions as you can see here.

Pre-study, we assessed leukocyte compatibility between donor and recipient based on HLA and B typing of both, based on an leukoagglutination cross match and measures of HLA antibodies using lymphocytotoxicity assay. We have accrued 25 donor/recipient pairs in this study, which we have closed. I only have data at the moment on six of these people, which I have shown for you here.

This is data showing the granulocyte component cell dose times 10^{10} for each day of transplant. This is the average cell dose. This shows you the increment in the average ANC at post-infusion hours 1, 4, 8, 12, 24, 36, and 48. I think that the increments that we observed on day +1 were reasonably good, but the increments that we observed on transplant days 4, 6, and 8 in my opinion were inferior to our prior results. And that occurred in spite of transfusing larger numbers of granulocytes.

I show for you here the results of the leukocyte compatibility test. Five of six of the donor/recipient pairs were not HLA and B matched. Lymphocytotoxic antibodies were detectable in four

of six patients. And the leukoagglutination cross match was zero or negative in all six pairs. So this preliminary data suggests to me that leukocyte compatibility may be an important determinant of neutrophil increments after transfusion of G-CSF mobilized granulocyte products, and it certainly at least provides an important clue that we really need to look at this area more carefully.

This is just a table comparing this data, the current data that I just discussed of autologous transplant patients who received predominantly leukocyte incompatible granulocytes. The component cell dose was 8.6 times 10^{10}, and the maximum mean peak ANC increment occurring was 796, and that was on only the day +2 transfusion. As you recall, the increments after that were substantially lower. If you compare that to our data that we published last year in Transfusion, these are allogeneic bone marrow transplant patients who received HLA matched granulocytes, again mobilized with G-CSF. In spite of transfusing a lower component cell dose than this current data, the ANC increments were substantially greater.

This brings to me the importance of considering doing red cell reduction of granulocyte components. Why? When selecting only leukocyte

compatible donors, the requirement of ABO compatibility reduces the pool of potential granulocyte donors. And the data from the last study suggested that we probably need to select leukocyte compatible donors. The objective of this study was to decrease the component of packed red cell volume to under 5 mls, a guideline established by the AABB, which we think will reasonably insure against hemolytic transfusion reaction in recipients of granulocyte components that are collected from ABO incompatible donors. And this is data we recently published in the Journal of Clinical Apheresis.

The trial design involved apheresis of granulocytes with hetastarch, and then after collection we performed gravity sedimentation of the component for 60 minutes, and then we transferred the red cell poor fraction to a sterile docked transfer bag utilizing a plasma expressor. The residual red cells were retained in the collection bag and were defined as the red cell rich fraction. And then we documented cell numbers and packed red cell volumes with each component or fraction. This data shows you that without manipulation of the component, the average granulocyte component

contains a packed red cell volume of about 25 mls, as Susan Leitman had discussed.

However, after component manipulation using gravity sedimentation ex vivo, the red cell poor fraction did contain substantially lower packed red cell volume, 6.3 ml on average. We observed that in these manipulated components, 40 percent of the red cell poor fractions contained under 5 ml packed red cell volume. Unfortunately, as I point out here, there is some cell loss. 20 percent of the granulocytes are lost to the red cell rich fraction using this technique.

So ex vivo, a hetastarch sedimentation as we performed in this study did reduce the number of red cells from the granulocyte components, but most red cell poor fractions still contained more than 5 ml packed red cell volume and thus were not acceptable for transfusion into ABO incompatible recipients. We are currently looking at extending the duration of sedimentation to 90 and to 120 minutes. At 120 minutes, we have been able to uniformly reduce the packed red cell volume to under 5 ml with this technique.

So I have thrown a slide up here for you to suggest an ideal trial design for prophylactic granulocyte transfusion support of transplant

recipients. The principle being the following: daily prophylactic granulocyte transfusions from onset of neutropenia to recovery from neutropenia. We would like to choose leukocyte compatible donors receiving a large dose of G-CSF. If you recall an earlier slide, this dose of G-CSF resulted in granulocyte components with 20 times 10^{10} granulocytes. So there is a reason for that, which I will get to. And then the donors undergo granulocyte collections on transplant days 2, 4, 6, and 8. The apheresis uses the higher IO setting of 35. Hetastarch is the red cell sedimenting agent. And then conceptually, from a practical perspective, it would be nice to split each granulocyte component, freshly transfusing half and trying to store overnight the other half, realizing that each component will contain roughly 10 times 10^{10} granulocytes. It is probably feasible to do that. The recipients would include transplant patients who were receiving adequate stem cell products, again as I have defined here, and also receive post-transplant G-CSF. And it would be nice to have some measure of choosing non-alloimmunized patients.

To conclude, first of all G-CSF improves the cell yield of granulocyte components collected from normal donors. Secondly, in allogeneic bone

marrow transplant patients, transfusion of G-CSF mobilized HLA matched prophylactic granulocyte components resulted in significant and sustained increments in the ANC, localized to sites of inflammation for up to two days post-transfusion, did not cause febrile reactions -- and I didn't mention this, but in our HLA matched granulocyte donor scenario, we have not observed febrile transfusion reactions. These components also resulted in significant increments in the platelet counts and reduced platelet transfusion requirements in this cohort of patients. And as many of you know, these granulocyte components contain 2 to 4 times 10^{11} platelets, which is equivalent to almost a unit of single donor platelets. In our experience in this patient cohort, we have been able to reduce platelet transfusion requirements in half with granulocyte transfusions. Third, antibiotic utilization was reduced in allogeneic PBSC transplant patients transfused with G-CSF mobilized HLA matched prophylactic granulocyte components on transplant days 3 and 6. Fourth, the preliminary data suggests that leukocyte compatibility was an important determinant of ANC increments after transfusion of G-CSF mobilized granulocyte components. And fifth, we really don't know the

optimal component cell dose and we really don't know the optimal frequency of transfusion of these products today. I think we need to learn that.

And finally, there is preliminary data from Phase II studies suggesting potential clinical efficacy of G-CSF mobilized HLA matched prophylactic granulocyte transfusions. I would suggest that we give some consideration for pursuing a Phase III trial at some point to really test this hypothesis.

I would like to recognize all of these individuals at Washington University for supporting these trials. I would also like to recognize Barnes Jewish Hospital, which also provided some financial support for these studies. Amgen has also been very kind in helping in doing these studies. And I would like to also recognize Gary Spitzer, who provided for me the initial encouragement to pursue these clinical trials back in 1992 and 1993.

Liana asked that I comment on what areas that I would recommend that we would pursue in terms of support of research in this area. I think that we really need to understand better the importance of leukocyte compatibility and incompatibility with G-CSF mobilized granulocyte transfusions. So I think that we really need to pursue that issue better and we need to define that issue better

before we pursue a Phase III trial. Secondly, the cell dose is really unclear. What is the cell dose we should be using in a Phase III trial? I don't think we understand that issue. How often should we give these granulocyte components. I think that we need to know answers to these questions before we do a Phase III trial. Red cell reduction may be an important area to pursue in research. Indeed if leukocyte incompatibility adversely affects outcomes, red cell reduction will be an important practical issue in order to expand the available donors that we could choose from. And finally, granulocyte storage. Again, from a practical perspective, we all would wish that we could store granulocytes. It is just, I think, an area that is probably worthy of considering. So I will just close with that point. Thank you.

DR. STRONCEK: Thank you for that very clear and insightful presentation. He ended with the right thing to talk about studying next storage. Tom Lane will now discuss storage considerations of granulocytes. Dr. Lane is Professor of Pathology at the University of California, San Diego. He is the Medical Director of their transfusion service and their stem cell laboratory.

DR. LANE: Thank you, David. And thank you, Liana, for inviting me to this conference. I have learned a lot and it has been very interesting. It is always a pleasure to talk about granulocyte storage. I will, of course, indicate that many of the people in this room contributed to the studies that I am going to summarize. Give me the first slide, please. Were I to actually go through all the data -- the first two slides are actually Dr. Harvath's. So if you will move on to the third slide in that carousel, that starts mine.

So were I to actually go through all the data, we would be here all afternoon. So I am going to summarize some data that has been collected over the years regarding granulocyte storage. I think the previous speaker has already answered this question for us, why should we store granulocytes or why should we know about the storage of granulocytes? And the answer to that is for one thing, as Jeff McCullough has said for years, granulocytes are inevitably stored for at least some period of time prior to transfusion. This relates to variables such as off-site harvesting, the testing requirements as Dr. Price talked about, who basically summarized my entire talk by saying that there are some defects associated with granulocyte

storage, transportation issues, and patient considerations. The patient may be receiving other forms of therapy that at least some people may consider incompatible with getting granulocytes at the same time, such as amphotericin. Whether or not that does make a difference. Or the patient may just not be able to get the granulocytes when they are ready in the transfusion service. And, of course, there are those of us who actually still do research on neutrophil function and storage becomes an important part of that.

But certainly for a clinical trial in which it may be of interest to store one portion of a granulocyte preparation, obviously you need to know whether or not they work. So I thought I would summarize this as others have, and I will go through this quickly. What the critical granulocyte functions are. Obviously, granulocytes need to stay in circulation for a period of time. They need to be capable of a certain amount of adherence but not too much adherence until they encounter an activated endothelium. They need to recognize the activated endothelium, a chemotactic gradient as indicated here by these little dots. They need to then migrate through a chemotactic gradient towards this happy bacterium that unbeknownst to him has been

opsonized by antibody and complement. They then need to engulf the bacterium and finally kill it. So these are critical granulocyte functions that must be accomplished.

Well, what do we know about the clinical efficacy of stored granulocytes. In fact, there are no studies out there that will attest to the clinical efficacy of storage of granulocytes. If you look at the seven studies that people refer to, those that were reviewed by Dr. Strauss, and look in the methods sections regarding storage, you will find that the granulocytes were transfused either immediately or within 4 hours or it is not specified. So at this time, there are no clinical studies that will attest to the efficacy of stored granulocytes. So that has left us then with looking at surrogate markers of the efficacy of the function of stored granulocytes. These can be easily broken into two general categories, in vivo studies and ex vivo studies. The former include granulocyte recovery, kinetics and survival, and distribution, either measured by isotopic techniques, migration into the buccal cavity as Dr. Price mentioned, or a more classical skin window chamber studies of a variety of different types. Or what have been measured more frequently because they are simply

easier and don't involve human studies or human manipulation, I should say, are looking at the cell numbers after storage, adherent function, chemotaxis function, and the other functions that I mentioned that are critical for granulocytes to do what they are supposed to do, which is to kill invading microorganisms.

Well, if you look at all these potential surrogate markers, which ones are really relevant to clinical efficacy? This is actually a fairly difficult question to answer. Let me back up for a moment and say I think clearly the number of granulocytes that need to circulate in vivo has been studied. There are classical studies by Bodie and co-workers in the late 1960's suggested that we need in the range of about 500 per microliter. Now that may be a facile measurement of something more complicated such as the total granulocyte storage pool, but there is at least evidence to suggest that if the circulating granulocyte level is above 500 that people are less susceptible to infection if they are functioning normally.

So apart from the cell number, what else do we know about how ex vivo function relates to the susceptibility for infection? At least -- I don't think you can come to any firm conclusions, but you

can get clues from, if you will, experiments of nature. The two that I have listed here are leukocyte adhesion protein deficiency of the beta 2 integrins and chronic granulomatous disease. This defect relates primarily to the adherence of neutrophils, LAD, and CDG, as you all know, relates to the failure of granulocytes to generate toxic oxygen radicals.

Now if you then separate patients with LAD into severe, moderate and mild and then look at various functions that are correlated with these clinical defects, you can come up with at least some generalities. And likewise with CDG. And rather than going through all this, I have summarized that on the next slide. All this data put together suggests that if skin window migration, primarily generated through looking at LAD deficient patients, is less than 80 percent of normal, this is associated with at least mild defects in resistance to infection. Likewise, in vitro chemotaxis defects, less than 70 percent of what passes for normal -- and anyone who has done this knows that this can be quite variable -- have been associated with infection. Adherence less than 50 percent has been associated with infection. Phagocytic activity of less than 40 percent, microbial killing of less

than 25 percent, and oxygen radicals, surprisingly you need very little in the way of oxygen radical generation to sustain normal microbial killing function.

So this gives us a clue then as to what kinds of surrogate markers are going to be useful to study when you look at granulocyte storage. Again, commenting on the fact that while perhaps the best studies are those related to in vivo function, that is to say do the cells migrate or localize to sites of infection, recognizing that those are difficult to do, most people are going to look at least initially at ex vivo functions. And then the next question you want to ask is well looking at ex vivo functions based on all of the relevant past experience, how do these functions fall out? And nearly all studies agree that chemotaxis, the migration of neutrophils, is the single most sensitive function during storage. It is the function that seems to have the earliest defects and is most sensitive to granulocyte manipulations in storage. And that seems to be followed by changes in adherence and microbial killing, followed in turn by changes in phagocytosis and oxygen radical generation.

So the next studies that I am going to summarize will focus to a large extent on chemotactic function. Because arguably if the cells won't migrate, then it doesn't matter if they have 70 percent or 100 percent oxygen radical activity.

So what are the important factors we need to look at when looking at stored neutrophils? What factors may affect the quality of stored neutrophils. Obviously the donors presumably will have normal neutrophil function and that is usually ascertained by whether or not they have a history of infection. Otherwise, they wouldn't be normal donors. Obviously related to the use of G-CSF, donor treatment with G-CSF or steroids. Collection techniques as has been summarized by Dr. Dale. There are differences in the efficacy of granulocytes based on whether they are collected using centrifugal techniques or filtration leukopheresis techniques. The concentration of the neutrophils in the component itself -- and this is perhaps the single most important thing I am going to say today. We are going to have to be careful in the modern age regarding the concentration of neutrophils in the bag as regards their storage. And others have shown that the concentration of

platelets too may affect the storagability of granulocytes.

Physical parameters of storage including temperature, agitation, the type of container, and metabolic parameters such as the anticoagulant used, the pH of the medium, which relates of course to the concentration of neutrophils, and the amount of glucose in the protein have been shown to affect granulocyte storage. And finally, the presumed effectiveness or lack thereof of preservatives such as growth factors. A letter to the editor regarding the use of gels to protect granulocyte function.

I may, in the interest of time, skip this slide since Dr. Dale has already reviewed the fact that cells collected by filtration leukopheresis can be shown up front to have diminished function and this simply illustrates that while cells collected by centrifugation leukopheresis at least after collection are normal -- and by the way, everything that I am going to talk about will be regarding donors not stimulated by G-CSF. So while cells collected by centrifugation leukopheresis are relatively normal, those by filtration are not and survive in storage very poorly.

What about storage conditions and how this relates to function of stored neutrophils? Well, very early studies by McCullough and co-workers have shown that different kinds of bags may affect storage. PVC bags appear to be the best of those looked at, at least at that time, and are certainly better than storing neutrophils in tubes. More recently, TOTM, and I can't remember right now what this stands for, or the CLX bags have been shown to provide improved granulocyte storage compared to other types. McCullough and co-workers looked at a variety of different kinds of anticoagulants and found some differences which I will show in a moment. Sedimenting agents have been largely shown not to affect granulocyte storage. I will talk about temperature, agitation, neutrophil count. Platelets I have already mentioned. Glasser and colleagues showed some years ago that platelets -- the presence of platelets diminishes granulocyte function after storage as a continuous variable, largely through the diminution, they found, of glucose content. Likewise, glucose content of the storage medium somewhere between 50 and 1,000 mg per deciliter appears to be optimal. Glasser and colleagues also showed that there is a requirement for optimal storage for protein and found that 1

percent albumin or plasma were equally effective, but not IgG. Other studies have shown a pH optimum in the range of 7 to 7.5 in preserving chemotactic function. I am going to just show you a little bit of that data now.

This is a study by McCullough and co-workers published quite a long time ago which showed for granulocytes stored at 4 degrees Centigrade in those days a slight advantage at 24 hours to cells stored in ACD or CPD anticoagulant compared to heparin or ion exchange. Not a great deal of advantage but a little bit.

For pH, McCullough and co-workers also looked at this as have others. Shown here is the initial pH of a storage medium now 24 hours at room temperature and the resultant ATP. This is chemiluminescence, the measure of toxic oxygen radical generation and chemotaxis using, I believe, a Boydian chamber technique. They found, focusing on chemotactic function, that a pH range between 7 and 7.5 was optimal. Either side of the chemiluminescence dropped off radically. This illustrates that chemiluminescence or the ability to generate toxic oxygen radicals in response to a phagocytic stimulus was somewhat more resistant to

changes in pH and ATP showed decrements in pH at anything other than about 7.5.

This slide summarizes two studies relating to the effect of agitation. It is interesting that there are differences. The first one by McCullough back in 1978 suggested that granulocytes stored at room temperature for 24 or 48 hours had a greater defect in chemotaxis if they were stored agitated, and Mary Clay was kind enough to remind me that this was using horizontal agitation, and these changes were more pronounced after 48 hours of storage. So there is an advantage to not agitating neutrophils according to these studies. But approximately 9 years later, some Japanese workers published in Transfusion just the opposite result using a somewhat different bag but the same kind of agitation, that is to say horizontal. They found chemotactic function better preserved in cells that had been agitated as opposed to left stationary. Now it is difficult -- looking at these two studies, it is difficult to make any sense out of this since most of the other factors relating to the granulocyte storage were relatively equal. What is of interest is that in the later study, the cells that were stored in a stationary fashion had a very, very marked defect in chemotaxis

after 24 hours, much greater than most other workers have found, and this leads one to believe possibly that there may have been something else going on in this study which didn't permit these cells to function as well. I just point this out to indicate that maybe this is something we need to look at again in view of this controversial data.

A great deal of work has focused on the temperature at which we ought to store granulocytes, and this is a study again by one of the leaders in this field, McCullough and co-workers, looking at room temperature versus 6 degree storage for 8 or 24 hours and looking at in vivo recovery and survival and localization, this percent skin window migration. To summarize this, these investigators found after only 8 hours of storage at either room temperature or 6 degrees, a significant benefit to room temperature storage in terms of overall granulocyte recovery, and this is percent recovery, compared with 6 degrees. And likewise after 24 hours of storage, again an advantage to room temperature over 6 degrees storage. The half-life measurements of granulocytes were a little bit more difficult to interpret, but again suggested an advantage to room temperature storage. But these investigators found, again, a marked advantage to

room temperature storage compared to 6 degrees in looking at migration of the cells into skin windows.

Some studies we did later suggested a possibility for the changes in granulocyte recovery comparing 6 degrees to room temperature storage. We looked at granulocyte adherence to endothelial monolayers after 24 and 48 hour storage of granulocytes at these two temperatures and found that cells stored at 6 degrees were somewhat hyperadherent, whereas those stored at room temperature for this period of time had relatively normal adherence function.

We also, as have others, found a significant benefit to room temperature storage compared to 6 degrees in terms of chemotactic function and this is distance migrated shown on the ordinate scale at 24 and 48 hours, room temperature versus 6 degrees, and this is random migration.

So this slide then summarizes a number of studies looking at room temperature versus 4 degrees Centigrade storage in granulocytes. Again, highlighting the differences, there are differences in adhesive function of cells stored at room temperature versus 4 to 5 degrees, improvements in chemotactic function or I should say less of a decrement in chemotactic function and less of a

decrement in the recovery after transfusion, and less of a decrement in skin window migration. So these and other studies led most people to the conclusion that room temperature storage for these unstimulated donors was superior to 6 degrees.

Someone asked the question today about the effects of irradiation. I have summarized here I think it is 8 different studies that have over the years looked at the effect of irradiation on granulocyte function. And I think we can summarize these by saying that over the range of irradiation, these are in gray, used to prevent GVHD, there really is no consistent effect on granulocyte function, either in cells that are collected freshly, as most of these were, or after storage for 24 or 48 hours here in two studies in which a wide variety of functions were observed. There is only one study that suggested that 50 Gray irradiation might decrease the nitroblue tetrozolium generation in granulocytes. And this is using a semi-quantitative technique. So I think the great weight of evidence suggests that irradiation does not affect these cells. Again, this hasn't been looked at in G-CSF stimulated donors, at least not published to my knowledge.

So using the "best of current techniques", what can we expect for granulocytes that have been stored for 24 or 48 hours. That means room temperature storage, not agitated. Well, we can expect about 50 percent recovery, which is not very different from normal -- I am sorry, 50 percent decrement in recovery compared with normal, which is decreased, whereas the survival of the cells will be close to normal. In vitro, that is to say in the bag, we can expect to recover most of the cells. Most of them will be there, between 99 and 88 percent. We can expect up to 20 percent decrement in in vitro chemotaxis and perhaps 10 percent decrements in microbial killing. So all of this data suggests then that these cells ought to function relatively normally once they are transfused. At 48 hours of storage that may not be the case.

The next and final thing that I want to talk to, and I am going to try not to go too much over time here, is I think important to the modern situation. Because we did some studies some years ago looking at the effect of granulocyte concentration, that is to say the number of granulocytes or their concentration in the bag, on the subsequent function of those cells. We looked

at three different cell concentrations, 2, 5, and 7 times 10^7 per ml. We stored the cells at room temperature for 24 or 48 hours in autologous plasma unagitated at room temperature. We found progressive decrements in ATP, in glucose, and marked changes in the pH of the surrounding medium. We found that -- well, let me summarize this. We found basically that you could prevent the changes in pH by adding 15 millimolar bicarbonate. Let's start with this box over here or this panel. Shown here are cells stored at -- let's see, it doesn't say it on here -- at 8 times 10^7 per ml, either in the presence of no additives, of glucose alone, of bicarbonate, or glucose and bicarbonate. And you can show that bicarbonate preserves the starting pH of these cells. Glucose will preserve the glucose content, but as shown here will not preserve the pH. And that likewise cells stored in the presence of bicarbonate, either with or without glucose, maintained their content of ATP, at least at relatively normal amounts.

Now how does this relate to their function. In another study, we looked once again at ATP content and the pH of cells stored at 2 or 8 times 10^7 neutrophils per ml, again in the presence of bicarbonate or without it. I think if we focus

on this panel, these are cells stored at 8 times 10^7 per ml in the absence of bicarbonate. You can see that their chemotactic function now using a Boydian chamber technique drops off rather remarkably. But in the presence of bicarbonate -- unfortunately I don't have the key here, so I am having to remember these old studies -- in the presence of bicarbonate, you can preserve chemotactic function at least for 24 hours, even at cells stored at this high a concentration. Likewise, you can preserve pH and ATP as I showed before. So the point of all this is that in the cells that are being generated and the granulocyte concentrates that are being generated today, if you translate the doses that you are giving in the range of 40 billion or so versus the volumes into which they are being collected, you are exceeding even what we studied here by two or three-fold in terms of overall cell concentrations. So if we found marked defects in pH maintenance and ATP maintenance and chemotaxis in cells stored at 8 times 10^7, then cells stored at 20 times 10^7 are probably going to be much worse off than we have here.

So I would hasten to add that we really do need to study how these cells will function after storage. And perhaps I would recommend then that if

one is going to try to store these cells, that you need to try to maintain the pH of the surrounding medium. That seemed to be accomplished by using 15 millimolar bicarbonate, at least in these studies. But I would not speculate whether that would be enough in cells stored at two or three times this concentration. I think that is an area that really needs to be looked at. And as an abstract to be presented later on shows, in fact the changes we found in pH here certainly are found in cells in stimulated donors.

So finally, I want to summarize here what everybody already knows. Over the past several years there have been a number of studies that suggest that certain cytokines can, in fact, prolong the storage life of neutrophils. Most of these studies were performed in cells stored at 37 degrees in tissue culture flasks or plates and looked only at so-called viability or trypan blue dye exclusion. So all one knew was that these cells were surviving longer. But more recently, Rex and co-workers have published in Transfusion some more interesting results. Again, these granulocytes were stored in culture dishes at 37 degrees, but they looked at some more relevant functions, in this case granulocyte chemotaxis to FMLP, and found that when

you store cells in this fashion for 20 hours, there is a marked decrement in chemotaxis that is preserved or prevented by the addition of G-CSF and gamma interferon. They also looked at superoxide anion generation at fresh and after storage, and again found a decrement with storage which was again prevented by G-CSF and gamma interferon. They looked at bacterial killing and found perhaps some improvement with G-CSF and gamma interferon in this decrement. Now this is percent surviving Candida. This is not really a convincing difference.

And then they also provided, as have others, an explanation for why these decrements in function were prevented by G-CSF. This is a slightly different organization here. We are looking at cells stored at 20 hours at 37 degrees as a percent of control, either unirradiated or irradiated with in this case I guess it is 5 Grays -- maybe that should be 50. They found, looking at apoptosis, that cells stored in this fashion with or without irradiation underwent marked apoptotic changes, as others have reported, but that the addition of gamma interferon and G-CSF with or without irradiation prevented apoptotic change.

Now I used to think of granulocytes as being end stage cells that didn't do much in the way

of protein synthesis and I really didn't understand these findings for quite some years, but I have been educated. Granulocytes are, in fact, capable of new protein synthesis and new messenger RNA synthesis, and I have just summarized some recent studies here showing that with these various stimuli, you can in fact get new protein synthesis, as indicated here, and this is accompanied by gene activation. And in fact in I guess it is the August issue of Blood, there is a new publication indicating that if you look at all the different species of messenger RNA that are made by granulocytes, there are over 700 different species of messenger RNA that are actively generated by granulocytes. So these aren't cells that are incapable of protein synthesis. And that perhaps explains how it is that G-CSF and some of these other stimuli can modulate the apoptosis of neutrophils. So I thought I would end here by showing that a variety of different cells can modulate or delay apoptosis -- interleukans 2415, GM-CSF, G-CSF of course, TNF alpha after prolonged incubation, glucocorticoids, et cetera -- while other functions, stresses, and agents may accelerate granulocyte apoptosis. Well, after being interested in granulocyte storage for 20 years, maybe this is the holy grail. Maybe delaying apoptosis in these

cells will permit them to be stored for a longer time.

But once again, I guess if I can say anything important here this afternoon, it would be that it doesn't matter whether the cells are apoptotic or not if their pH is 5.5 and they are dead. So I think with the modern collection techniques, we need to be mindful of the concentration of granulocytes in the medium and do something about that. And then in the future, it may be possible to prolong or permit longer storage using some of these agents. And in response to Liana's question, I am going to sound like a broken record. I think we need a trial of the efficacy of granulocytes stimulated by G-CSF in donors before we can really know whether they are working. I would be -- I think we need to study granulocyte storage, but I would be hesitant to store granulocytes in such a trial. I think we have no current knowledge that stored granulocytes, at least beyond 6 to 8 hours, really work, and I would be concerned at least in a major trial that it would not be beneficial to the potential efficacy of such a trial to include stored granulocytes. I think that needs to be studied separately. Thank you very much.

DR. STRONCEK: Thanks, Tom. It sounds like once granulocytes are proven to be effective, we will be busy for quite a while figuring out the best way to store them. The next presentation will be by Conrad Liles, who will talk about in vitro assays predictive of product function. Dr. Liles is an Assistant Professor in the Division of Allergy and Infectious Disease of the Department of Medicine at the University of Washington, Seattle.

DR. LILES: The title of my talk is as introduced, and I thank the organizers for inviting me to this workshop. It is in vitro assays predictive of leukopheresis granulocyte product function. It is a little bit of a difficult task because that is what people have been talking about the entire day, but I am going to try to talk about our studies in evaluating leukocytes or granulocytes that are mobilized with G-CSF and then also those granulocytes during storage and storage plus or minus the readdition of G-CSF ex vivo.

First of all, I wanted to talk about -- you have seen this slide before, but this is why we chose a regimen of 300 mcg of G-CSF and 8 mg of dexamethasone to stimulate our donors in our granulocyte collections. So we proceeded to use this regimen because it seemed to give the maximal

ANC and it seemed to give it within 12 hours. The addition of dexamethasone significantly increased the maximal ANC induced by the dose of G-CSF alone. The greatest mobilization was with 600 mcg of G-CSF and 8 mg of dexamethasone. This drug regimen at least overall was relatively well tolerated by the normal volunteers that we used in this study.

The protocol involved treatment, as you have seen earlier, with 600 mcg here and collection by leukopheresis. And then the measurements were cell numbers and morphology, immunophenotype analysis by flow cytometry, chemiluminescence, bacteriocidal function, and then blood and tissue kinetics in these cells immediately after collection. The collections were from 5 donors and you can see here that the mean number of cells collected was 77 times 10^9 cells. That is after a starting neutrophilia of 28,700 in the donors.

First of all, we looked at chemiluminescence. This just shows you the luminol-enhanced chemiluminescence activity of the neutrophils in these collected granulocyte fractions. We evaluated baseline, that is, these were -- after stimulation of these donors, we went ahead and looked in their venous blood and looked to see what their chemiluminescence activity was in

response to PMA. We also looked at the baseline activity right here prior to collecting the actual neutrophils and stimulating the donors, and then we also looked at the leukopheresis product. What we found is that after stimulating with G-CSF and dexamethasone, you actually saw a priming effect of the stimulation procedure, so there was greater chemiluminescence activity or greater oxidative burst potential in response to PMA after giving G-CSF and dexamethasone. But then if you looked at the cells after leukopheresis, there is actually a slight detriment. So the leukopheresis procedure per se actually impairs the subsequent oxidative burst, but still it was greater than just cells obtained prior to the G-CSF stimulation. So our conclusion was that the product here had good respiratory burst activity and would have potential activity in fighting or in having microbicidal activity if retransfused.

We did look at bacteriocidal activity and the staphylocidal assay ex vivo. We found that the leukopheresis PMNs that were obtained after G-CSF and dexamethasone stimulation were just as effective as baseline PMNs in terms of killing the Staph aureus. So the bacteriocidal activity appeared to be fine when immediately collected.

When we looked at immunophenotype analysis of these cells versus baseline cells, you can see that in terms of L-Selectin, there is a slight decrease, about half or 50 percent decrement, in the L-Selectin expression on these cells as compared to baseline PMN's. CD11B was about doubled. CD18 was also doubled. We didn't really see a large induction of CD14 at this dose of G-CSF. And then CD16, which is FCgamma R3, was actually decreased on the leukopheresis cells. CD32 or SCgamma R2 was actually about the same. And then we did see an induction of SCgamma R2 or CD64, about a doubling there. What we concluded from this is that the leukopheresis cells did have a slightly different immunophenotype, but it was a favorable immunophenotype, and one -- given that we had expression of the SCgamma receptors -- one in which we though the cells would be effective in terms of normal host defense function.

We then went on to look at the in vivo kinetics of these cells when retransfused. David already mentioned this earlier today. When we retransfused the cells, we saw that they had a prolonged half-life. What is not shown here is that these cells not only circulated with the prolonged half-life, but they could get to inflammatory tissue

sites. In other words, we did recover these cells from the buccal mucosa when they were relabeled and then retransfused, and they also migrated to skin windows effectively. So that these cells not only migrated, but they could also migrate to potential inflammatory sites.

So our results overall show that we could get a good yield of neutrophils from donors stimulated with one dose of G-CSF and dexamethasone, and then the leukopheresis performed 12 hours afterwards. The respiratory burst activity -- I didn't show you all the other respiratory burst activity to different stimuli, but it was more or less normal or at least there was significant activity to the point that the cells would have microbicidal activity. Bacteriocidal activity, at least against Staph aureus, was normal. Immunophenotype showed increased CD11B and CD18 and also an induction of CD64. And the kinetics showed an increased blood half-life, but also the ability of the cells to migrate to tissue sites. So it appeared overall that these cells obtained from individuals after a single dose of G-CSF and dexamethasone appeared to be functional and would be viable candidates in a neutrophil transfusion program.

So just to summarize again, G-CSF plus dexamethasone allows much improved neutrophil collection and the treatment is relatively well accepted with a few adverse effects. Metabolic and bacteriocidal functions are preserved and the cell half-life is prolonged. And we have concluded that transfusion of these cells to neutropenic patients may be useful.

In the second part of this talk, I really want to talk about storage, because that is really the frontier at this point in terms of improving the program. As you have heard, many blood banks would not have the capability of collecting on weekends or routinely on weekends. So the ability to obtain cells and then store them for 24 to 48 hours would greatly facilitate any sort of neutrophil transfusion program. So we wanted to see whether or not with the knowledge that we had concerning apoptosis and other storage variables, whether or not we could come up with a regimen to show effective storage for 24 to 48 hours so that this could be adopted if we were to have neutrophil transfusion programs instituted on a nationwide basis.

The factors compromising the clinical utility of granulocyte transfusion therapy have been

discussed, but one of the major factors, as was just discussed in the talk preceding mine, is that neutrophils rapidly undergo apoptosis during storage in vitro. And as they undergo apoptosis, functional activity declines. Granulocyte products obtained by leukopheresis are currently transfused into the recipient as rapidly as possible without storage. As soon as that granulocyte product is obtained, it is usually shipped and transfused as rapidly as possible without any storage whatsoever. And this certainly would hamper any sort of program to be instituted nationwide.

If we could get effective storage of granulocytes, then we could be able to obtain a leukopheresis preparation and a granulocyte preparation on a Friday and then use it through the weekend and then get another donor on Monday, and it would greatly facilitate the ability to maintain an absolute neutrophil count in a recipient through the weekend without undue stress in a blood bank program.

The factors that are known to decrease neutrophil apoptosis during storage ex vivo include anaerobic environment. We actually looked at this and if you really do store isolated neutrophils, that is neutrophils that you have obtained by

venipuncture and then culture them or maintain them in RPMI plus 10 percent fetal calf serum ex vivo, the anaerobic conditions do prevent apoptosis and do significantly prolong isolated PMN survival. However, when we looked at the leukopheresis product, that is, the product obtained after G-CSF and dexamethasone, centrifugation leukopheresis, and then tried to look at the effect of the anaerobic environment on that leukopheresis product, we found no significant effect. So you can't translate findings that you might see with isolated PMNs with the actual survival of PMNs in a granulocyte product. So anaerobic environment did not appear to be a viable option to maintain cells during storage.

We also found that reduced temperature also reduced apotosis of neutrophils during storage or during maintenance ex vivo, but if we tried to translate this at 4 degrees into storage of the leukopheresis product, we found that this was impractical. At 4 degrees, there was a lot of clumping of leukocytes that could never be retransfused. However, when we looked at 10 degrees, we found no significant clumping of leukopheresis products at 10 degrees. So we subsequently tried to look at whether or not 10

degrees was more effective than room temperature, and I will get to those studies.

It is also known that cytokines, especially G-CSF and I suppose GM-CSF, are most effective at decreasing neutrophil apoptosis. So we wanted to look to see whether or not the readdition of G-CSF ex vivo to the leukopheresis product would further prolong neutrophil viability in addition to just the viability that we could obtain by reduced temperature.

Corticosteroids are also known to decrease neutrophil apoptosis, but we didn't want to add additional corticosteroids to the product that we obtained. And, of course, we couldn't add other agents like LPS which are also known to decrease neutrophil apoptosis.

So we thought the best way to try to study prolongation of neutrophil survival in the leukopheresis product -- and this is the leukopheresis product obtained after G-CSF and dexamethasone -- was to look at reduced temperature and also the readdition of G-CSF. This appeared to be most practical.

So the protocol for our study was to stimulate donors with 600 mcg G-CSF subcutaneously and also 8 mg of dexamethasone orally. Then

centrifugation leukopheresis was performed 12 hours after stimulation. Then we looked at storage or baseline and at 24 and 48 hours. We looked at room temperature as one condition. Room temperature plus the readdition of G-CSF with 100 nanograms per ml to the storage bag. We looked at 10 degrees and then 10 degrees plus G-CSF added to the storage bag.

Then we looked at the following parameters. We looked at white counts and differential, respiratory oxidative burst activity, immunophenotype, staphylocidal activity using a conventional four plate assay of killing of Staph aureus, and also fungicidal activity. In terms of fungicidal activity, we didn't look at what has usually been looked at and that is blastochlamydia killing. We actually looked at hyphae damage, which is more relevant for the clinical situation, which I will get to.

Now in terms of storage of this product, when you look at the ANC of the product, you can see that it really doesn't change regardless of what storage condition that we had. This is fresh product here and you can see that there is no significant difference if the product is stored at 10 degrees or at room temperature, or if it is stored in the presence of G-CSF, which is shown not

on this slide but another one right here. So under any condition at reduced temperature, whether it is room temperature or if it is at 10 degrees, you get preservation of the ANC in the product, and the addition of G-CSF did not appear to affect the subsequent storage.

Now next when we looked at the respiratory burst activity, we used luminol-enhanced chemiluminescence, which is a rapid and sensitive way to look at the respiratory burst and allows for a kinetic analysis. We used a variety of stimuli. We used PMA as a soluble stimulus. We also looked at opsonized zymosan as a particle stimulus, and its activity is primarily related to its ability to bind and then to be engulfed in the CD11B18. And then we also looked at FMLP, and FMLP of course binds to a cell surface receptor. I am just going to show you the PMA and opsonized zymosan results, just because it gets repetitive if we keep on going through it.

Here if we looked at the storage of this product, and this is at 24 hours, with the stimulus being PMA with and without G-CSF at the various temperatures, you can see that the product is always better in the baseline activity. It doesn't appear to matter significantly, at least at this level, whether or not the product is stored at room

temperature or at 10 degrees. It also, although there appears to be a relative greater benefit here at 10 degrees as opposed to room temperature, this is probably not clinically significant because the baseline product is certainly no better than here and this probably reflects that initial diminution in the product that I said that we saw earlier. That is, after the product is first collected, it appears to be relatively refractory to a secondary stimulus, but it regains in response to that stimulus. So if we were to look at this, we would say in terms of overall oxidative capacity, it is relatively well preserved either at room temperature or at 10 degrees and the readdition of G probably didn't make any difference.

Similar effects were also seen at 48 hours. We won't dwell there. And it is also seen with opsonized zymosan as a stimulus. Here you see the baseline product and then under any of these storage conditions at 24 hours, you see enhanced activity in response to opsonized zymosan. You still see this enhancement at 48 hours. So overall, we can say under these storage conditions here, oxidative capacity appears to be preserved and the readdition of G-CSF may not be necessary to retain that property of the cells.

Now we also performed immunophenotype analysis of the neutrophils during storage. We looked at adhesion receptors at CD11B and CD18 and also L-Selectin. We also looked at the three IgG CD receptors, CD16, CD32, and CD64. We also looked at CD14. I would just like to show you the CD16 and CD32, and CD64 data for simplicity, because I don't want to have to go through all the other ones here. The major point here is that in terms of CD16, CD16 declines as cells undergo apoptosis. What you can see here is that although there is a slight decline, CD16 expression is maintained throughout the storage period or through 48 hours whether or not the cells are maintained at 10 degrees or at room temperature and whether or not G-CSF is present or absent. So what we would conclude is that storage at reduced temperature with or without G-CSF maintains CD16 expression, retains CD32 expression, and also retains CD64 expression. So that reduced temperature of this leukopheresed product during storage maintains cellular viability and a favorable immunophenotype in terms of FC receptor expression. A favorable immunophenotype was also retained if you looked at CD14 expression or in terms of adhesion receptor expression.

Now I am not going to show the bacteriocidal activity here, because the bacteriocidal activity was always greater than 95 percent throughout the study period. So bacteriocidal activity was always intact regardless of the storage condition. But what is more important when we are considering the microbicidal activity of a storage product is actually a fungicidal activity. Because if we were going to envision a clinical trial, one would be most concerned with serious fungal infections in our neutropenic patients. That is really where the real problem is in terms of the oncologic and infectious disease standpoints.

I just wanted to emphasize the importance of opportunistic fungal infections in neutropenic patients. Prolonged neutropenia or abnormal neutrophil function are the major risk factors for opportunistic fungal infections. These opportunistic fungal infections now represent the major cause of infection-related mortality in bone marrow transplant or marrow transplant patients. And of these opportunistic fungal infections, invasive Aspergillosis and Candidemia are the most common opportunistic infections or mycotic

infections in these marrow transplant patients in patients with prolonged neutropenia.

Our experience at the Fred Hutchinson Cancer Research Center in Seattle from 1992 to 1996, which includes the period -- and this is important for trial considerations and while you really can't probably rely on historical controls -- and that is because our infectious disease prophylactic regimens have changed significantly and thereby impacted outcome and survival of patients as compared to years past. From 1992 to 1996, this reflects the experience during fluconazole and ceftazidine prophylaxis. At the Fred Hutchinson Cancer Research Center during this period, only 40 percent of patients who developed fungemia during neutropenia had clearance of fungemia within 10 days and survived for four weeks. More importantly or just as important, less than 30 percent of patients during this period who developed invasive mold infections during neutropenia survived for 12 weeks. This just emphasizes the importance of these infections in this patient population. We can treat most bacterial infections fairly effectively nowadays. The problem of CMVs still exist, but our methods to control CMV problems are much better than they were 10 years ago. But fungal infections

remain a major problem and it is a problem that now defies current antimicrobial prophylaxis and one that is really a frontier, I think, of oncology and infectious diseases.

Now in terms of the fungicidal activity of the granulocyte product during storage, we wanted to look at hyphae or pseudo-hyphae killing. The reason being is that hyphae of true molds like Aspergillus or rhizopus species or pseudohyphae of Candida species are the predominant tissue forms of opportunistic fungi during invasive infections. Usually people look at Candida or blastocandida, which are easier targets. The hard target are the hyphae and pseudohyphae, so we really wanted to evaluate whether or not the cells stored under these conditions could actually have activity against hyphae and pseudohyphae. To do this, we employed an XTT assay which measures leukocyte mediated damage to hyphae or pseudohyphae in vitro.

What we found here -- we will first look at Candida Albicans. It is that we looked at this product that was stored at 10 degrees without the addition of G-CSF, and we looked at neutrophils obtained or the buffy coat of patients prior to stimulation or of donors prior to stimulation, then at baseline, and then at day 1 and 2 of storage of

the product. So this is the buffy coat of individuals prior to getting G-CSF and dexamethasone. This represents the activity of the leukopheresis or the granulocyte product after leukopheresis on day zero after individuals received G-CSF and dexamethasone for stimulation. And then this is the storage of the product at 10 degrees without the addition of G-CSF. Then we looked at two effector to target ratios, the effector cell being a leukocyte in the granulocyte product, and the target being the pseudohyphal form of Candida Albicans. And what you can see here is that the activity against the pseudohyphae is maintained throughout the storage period. We see good activity that is maintained throughout the storage period. So these cells even after 48 hours can still mediate activity against Candida Albicans.

Similarly, activity is fairly well maintained against Aspergillus fumigatus hyphae, a very tough organism or a very tough form of the organism to kill. So that we see especially at the 10 to 1 E to T ratio good maintenance of activity throughout the storage period. It is certainly greater at day 1 than day 2, but we still have a significant level of activity at day 2.

So what can we conclude from these studies? First of all we can conclude that the granulocyte product obtained by centrifugation leukopheresis from donors stimulated with G-CSF and dexamethasone retain significant functional activity when stored at reduced temperature for 24 to 48 hours. From these studies, we could not conclude whether or not 10 degrees was better than 22 degrees. In many of the assays, 10 degrees looked to be slightly better, but I don't know if that would be clinically significant. So I think that just reduced temperature -- in other words, storage at room temperature or 10 degrees would be superior to storage at 4 degrees or at 37 degrees. Also, the addition of exogenous G-CSF to this granulocyte product provides at best modest benefit and probably would not be necessary to maintain granulocyte products for 24 to 48 hours.

So what are the important questions I think for future research in this area? Well, first of all, we have done our ex vivo or in vitro analysis. Now I think it is important to determine does the stored granulocyte transfusion product function with appropriate in vivo kinetics when transfused. We should establish this to make sure

that products could be stored if we were going to do a clinical efficacy trial.

The most important question, though -- and this is to prevent the unwise use of granulocyte transfusions in the future, and that is actually to perform an appropriate randomized control multi-center clinical trial. We have to know what the appropriate clinical indications for granulocyte transfusion therapy are. We can't really extrapolate from historical controls because the practice has changed. We have better antimicrobial regimens. So really some sort of controlled multi-center clinical trial is necessary to determine what are the appropriate clinical indications and the specific clinical indications for granulocyte transfusion therapy. We have to know is it going to be effective for invasive fungal infection or invasive Aspergillosis for mucal mycosis. Will it be effective for fungemia or Candidemia? Will it be effective for bacteremia in the neutropenic host? I actually don't think that we will see a benefit here just because bacteremia in the neutropenic host is usually fairly well treated now with the antibiotics that we now employ in clinical practice. Then also another area, as David mentioned earlier, was neonatal sepsis. We have to determine the exact

specific indications for neutrophil transfusion therapy. If we don't, it will be used maybe inappropriately in situations and actually could probably cause much greater harm than good and also be an unwise use of resources in the future. Only through a randomized controlled clinical trial can we determine the appropriate indications and avoid that unwarranted use.

So I would like to acknowledge my other collaborators during these studies, David Dale and Tom Price. Milton Gaviria is a fellow that works with us and he has been doing a lot of the antifungal assays. Then Ellen Roger is a technician who has been working with David Dale and myself for a long period and she has been working with the granulocyte storage for the last several years. Thank you.

DR. STRONCEK: I'd like to have the speakers from this afternoon come up and we can answer questions. Dr. Leitman?

DR. LEITMAN: Thank you. I have a question for Dr. Liles. In a slide shown by you and earlier by Dr. Dale on the kinetics of in vivo recovery of autologous labeled G-CSF mobilized granulocytes, the first column had to do with the recovery. And the in vivo recovery, if I read that

right, was lower in the G-CSF treated products, a 65 percent in untreated versus 31 percent, although the half-life was twice as long, 9.6 versus 20 hours. Could you comment on that decreased recovery?

DR. LILES: Well, actually -- Tom, would you want to comment? Or Tom or David, do you want to comment on that?

DR. DALE: The recovery is lower. That is, if you transfuse cells that have been collected by leukopheresis, you don't get the same percentage of those cells circulating initially. I think there is an element of damage to the cells that occurs with collection. And several people have commented that the one-hour increment in the counts are not necessarily the highest. And Tom and I found years ago that if you collect cells by leukopheresis and transfuse them, sometimes the counts go up and actually cells that initially marginated will enter the circulating pool. Those are the extrapolated values you would get at the initial time of transfusion. So I think that the values are lower than normal, but I think the cells probably do recover some function from being back in the warm, healthy body.

DR. LEITMAN: So those are one-hour recoveries. But from data presented by all of us

here today, it looks like the 4 or 8 hour post-transfusion count is higher.

DR. DALE: That is right. These are extrapolated based on radioisotopic. In normal people, not in neutropenic people, you have to use an extrapolated value based upon isotopic labeling. But it is -- would be correct if you could measure the recovery at four hours later approximately. I suspect it would be higher. Does that make sense?

DR. LEITMAN: Yes. I have another question. This is for Dr. Adkins. In your last trial -- you went through a lot of different trials -- you are giving allogeneic donors 15 mcg per kilo of G-CSF. I want to point out that anytime you exceed 10, that is two subcutaneous injections because most nursing standards do not allow you to exceed 1.5 to 2 ml per single subcutaneous dose, which doubles the discomfort to the donor to get two subcutaneous shots rather than one. The increment in your yield was 15 mcg per kilo versus 5 mcg per kilo. It was not very great. I think you had 10 times 10^{10}, whereas Seattle and NIH are getting around 8 times 10^{10}. And in every study I have seen, there is a dose-dependent increase in adverse effects in the donor. So could you justify why you are using 15 mcg rather than the lower dose?

DR. ADKINS: Well, actually in most of the patients -- the vast majority of the patients in our clinical trials, of which several you have seen, we have used either 5 or 10 mcg per kilogram. We were interested in defining whether or not there was a dose response effect in terms of component yield. So we have a very limited number of people that received 15 mcg per kilogram. In the autologous transplant trial, which was the latter one that I mentioned, we were giving 10 mcg per kilogram in that setting. So I am not certain that we are going to necessarily pursue doing 15. I think you can make an argument. If you are going to use a single donor to donate granulocytes for one patient over a course of a week or so and if you are thinking of the strategy of storing overnight a portion of the components, you can make an argument for using a higher cell dose given that you get greater yields with that approach. I mean, that is just a strategy that one might take and that is something that we are kind of looking at at Washington University. I think that I agree with you about the issue of toxicity. You know, we -- in the way we do this, the donors clearly express a much greater problem with toxicities during the phase of giving growth factors to collect stem cells as opposed to the time

in which we give the growth factors to collect the granulocytes. I don't that the problem with toxicity is a very big problem when we give the growth factors to mobilize the granulocytes. Their biggest complaints occur the week prior when we are trying to collect their stem cells, as I discussed earlier.

DR. SNYDER: Ed Snyder from Yale. Just a couple of practical aspects. We had done some work many years ago with stored granulocytes at room temperature looking at the ability to put them through an electromechanical pump because many of the oncology units were doing that to decrease the flow rate and yet make sure they went in in an appropriate time. So studies, if they are going to be repeated with the G-CSF, that might be a very practical point to look at to see if the mechanical shear stresses don't have a negative impact on granulocytes that go through the pump. Because some of them can chew up the red cells. But we didn't see any problems at that time without G-CSF several years ago.

Another study that we did was to look at the effect of the granulocytes on the platelets. Because with all due respect to the neutrophils, we think platelets are also beautiful cells that we need to care for. And what we found was that stored

for 24 hours at room temperature, there was a decrease -- if I can remember this and I would have to go back and look at the paper -- a decrease in GP1B on the platelet, which we thought was due to release of neutrophil enzymes during storage. So if the FDA is going to consider neutrophils with platelets in them, then someone needs to study the effect of storage, certainly G-CSF stimulated granulocytes on platelet function or whether the platelets should be removed from the storage separately if they can co-exist together in the bag. So I just mention those for the record.

DR. DIAZ: A quick question for Dr. Liles. If I interpreted your slide correctly, the actual kinetics of the response at 48 hours or at some of the late time points seem to be totally different from the normal classical response of up and down in 15 minutes and then totalling up to 60 minutes at time zero. Can you explain that or did I just read it wrong?

DR. LILES: Which one? The stimulation with PMA? You mean the chemiluminescence?

DR. DIAZ: Yes.

DR. LILES: I don't have a good explanation of why that is in terms of why it is flattened initially. Is that what you are saying?

DR. DIAZ: Yes.

DR. LILES: And then it rises out. No, I don't have a good explanation of why. It is just that that was a constant observation.

DR. DIAZ: Okay, thanks.

AUDIENCE MEMBER: This is a comment for Tom Lane. I liked your presentation in reviewing all of the details about storage. I think, though, when we consider function, we can't go back to and rely on these congenital defects in neutrophil function to give us some idea on what we should expect or what we should shoot for. An example of this is that you had stated that we needed to have perhaps 10 percent -- at least 10 percent or around 10 percent oxidase activity. It depends on how you look at it. If you look at the kind of classic patients with chronic granulomatous disease, they have no activity. If you look at variants, that data of 10 percent comes from variants. And if you have perhaps 10 percent normal cells, then you will have normal function. That is very different than a patient that I have who on a good day all of her cells have 20 percent activity and she is always having problems with infection. And it should remind us that one of the things that we have to evaluate in terms of function is whether if we see

decreased function or increased function, whether that is related to a subset of cells or whether it is all cells with lower function or just several different populations of the function.

DR. LANE: Yes. I think your point is very well taken. That was just meant to give general information. The other point that I neglected to make is that certainly in the presence of multiple defects in function, even minor defects may take on a lot more importance. So I think that needs to be kept in mind as well.

DR. STRONCEK: Along those lines, though, I think the issue on storage is very confusing. I agree with Tom Lane's summary of the literature that probably if you are going to do one functional assay in the laboratory on stored granulocytes, it should be chemotaxis. But that said, the literature suggests that storing granulocytes at less than room temperature might preserve a lot of function but not chemotaxis. I guess my question for you, Dr. Liles, is have you looked at chemotaxis of your cells stored at 10 degrees?

DR. LILES: No. You know chemotaxis assays are the most probably variable of all the assays and that is always a problem. With newer

sort of techniques, it is possible that you could. It might be less laborious. But I think actually the best test is actually to do the in vivo study that we were talking about and to retransfuse and to see whether or not you can get proper in vivo migration to the potential inflammatory site, meaning the skin window or to the buccal mucosa. That is really the real test, even better than in vitro chemotaxis. So I think that illustrates the point. Really we have to look at these cells when retransfused in vivo to see whether or not they can adhere to vasculature or to the endothelium and then migrate through the endothelium to an inflammatory site. That will be most important prior to conducting a large clinical trial to make sure that stored granulocytes can migrate to an inflammatory focus.

DR. STRONCEK: Conrad, a minor point. When you talked about bacteriocidal activity, was that at a one to one ratio? Did you look at different infectious ratios?

DR. LILES: Dan, we didn't look at different ratios. It was at a one to one at that point. So we didn't look under a stress situation, so to speak.

AUDIENCE MEMBER: Conrad, I think you probably mentioned this, but I probably missed it with all the data. When you collected these granulocytes and stored -- first of all, you collected them on the CS3000 in what volume? And then when you stored them, you just stored them as a product? You didn't isolate the granulocytes?

DR. LILES: Exactly. So they were the standard conditions that you saw earlier when Tom Price discussed it. So they were stored under those same conditions and under those same collection parameters -- yes, COBE.

AUDIENCE MEMBER: COBE. All right. So what kind of volume do your granulocytes -- what is the final volume?

DR. LILES: The final volume is more like 300 cc or so.

DR. LANE: Again, to ad nauseam. I am really concerned about the cell concentrations, particularly when these cells are stored at room temperature, and I think that is one of the first things that people should look at. And I guess you are going to present some work on that this afternoon.

AUDIENCE MEMBER: I would like to reflect on a comment that was made before and

actually second that. That is we seem to be coming down to two basic issues. One is a clinical trial to look at the efficacy of granulocytes, and the other big area is going to be to look at storage. And I think probably we are not going to be able to look at storage -- we are not going to be able to look at them both at one time. It would probably be wiser to not store for a clinical trial and then come back later when some basic work is done on storage techniques and look at that.

DR. LILES: Could I also make a comment. I think also the issue of alloimmunization is very important. Because when I am approached by an oncologist regarding the possibility of granulocyte transfusions from somebody in the pre-bone marrow transplant setting, the question is always will giving granulocytes from community donors obviate the chance for a successful graft later. And we really don't have good information to say whether or not that is the case. And that is always going to be a hindrance, I think, to the use or this practice unless those data are available. So the issue of alloimmunization and its clinical importance is still one that is out there and is worth further investigation.

AUDIENCE MEMBER: With regards to alloimmunization and the storage issue, has anybody on the panel considered cryopreservation, which would allow you to have autologous donations prior to treatment?

DR. LILES: We tried cryopreservation by different techniques and you get a gelatinous mess.

DR. LANE: I am sorry, I meant to comment on that and I didn't. It is very interesting if you review the literature on cryopreservation of granulocytes in that you usually find one or at most two publications by the investigative groups. Most of the people who study cryopreservation disappear from the face of the earth and are never heard of again. And that always worried me. I think there may be actually a few of us around. But cryopreservation is a technology that is so far away from being practicable for these kinds of cells that it is a major area that would be wonderful if someone could find out how to do it, but so far we are very far away from that.

DR. DALE: I would just add that what happens with the best cryopreservation methods now, if you look by electron microscopy at the cells, they blub. And it is probably that the freezing, even in the best of circumstances, disrupts the

membranes of the granules and you get some damage to the cytoplasmic components of the cells. We actually in the last couple of years have taken this far enough to measure chemiluminescence of thawed cells after freezing, and there is a little activity there. But it is really much diminished. And I don't know where the breakthrough will come in getting the cryopreservative into the cell to somehow preserve its many very fragile parts, but I would not be optimistic either. Really the hope is for two or three days of storage.

AUDIENCE MEMBER: Well, I think one of the issues is the clinical situation. If you look at something like the red cells where you have the ability to have wash steps and post-thaw treatments, you might be in a much better situation than if you needed something that would be directly transfusable. Because there are new cryopreservation programs now that are looking at water structuring molecules that cross the membrane that are transfusable and nontoxic that can, in fact, stabilize organelles and other components. So while it hasn't been shown recently with granulocytes, I think there has been some advance in cryoprotectant technology that may allow them to be applicable in this situation. It certainly appears

that we have a much hardier cell population now also in terms of these stimulated programs and the ability to reduce the amount of red cells and platelets in the bags.

DR. LANE: I'd agree by saying that there is a great opportunity for good work to be done in this field.

DR. STRONCEK: If there are no more questions, I guess that concludes this part of the presentation. We will have abstracts, I guess.

CHAIRPERSON HARVATH: We have three abstracts. Dr. Diaz is here from La Jolla, and Idun Pharmaceuticals is going to speak on the preservation of neutrophil viability through inhibition of cast base dependent apoptosis. Then after he speaks, Dr. Stroncek is going to present a couple of abstracts of their work here that he has done in collaboration with Dr. Susan Leitman and colleagues at the NIH.

(Whereupon, at 2:59 p.m. off the record until 3:03 p.m.)

CHAIRPERSON HARVATH: We will let David Stroncek present his abstracts first. They are going to try and fix the bulb. Dave?

DR. STRONCEK: I'd like to thank everybody who is still here at the end of a long two

days of meetings. I would like to talk about some studies. They are preliminary studies that we have done looking at first some of the safety aspects of giving G-CSF and dexamethasone to people donating granulocytes. I have had a lot of experience giving G-CSF to normal donors, particularly with peripheral blood stem cells. And the question comes up during every lecture similar to the ones we got today about how safe is this really to be giving donors a drug.

So with that in mind, I thought it was worthwhile looking very carefully at granulocyte donors to determine the effects of G-CSF on them. We know from studies on giving G-CSF to stem cell donors, and we heard some of this this morning, that there is a marked effect on neutrophils giving G-CSF. After the G-CSF is given and stem cells are collected, platelet counts fall. The platelet counts fall not only due to the dropoff from the apheresis collection, but there seems to be some direct suppression of platelet production by G-CSF. In addition, there is a transient neutropenia and lymphopenia that occurs after the collection of G-CSF mobilized stem cells. We also know in stem cell donors there is a marked increased in alkaline phosphatase and LDH, slight increases in uric acid, and falls in potassium and bilirubin. These falls

are transient but they do occur predictably in donors. As you have heard this morning or today already, both stem cell donors and people given G-CSF to donate granulocytes do experience headaches, bone pain, myalgia, and fatigue.

The question I wanted to focus more on was what happens with blood counts and blood chemistries in donors given G-CSF. In specific, one question that comes up is because we are using kind of a small group of people that we have talked to that we have asked to give G-CSF, we tend to use them over and over again to donate granulocytes. One question that I have asked and we have asked is how often can someone safely donate granulocytes. I am not sure what the answer is, but I do know I would like their blood counts and chemistries to be normal before we give them G-CSF or dexamethasone again and collect granulocytes.

So with that in mind, we designed this study. We mobilized granulocytes three different ways. Either with dexamethasone, G-CSF, or dexamethasone plus G-CSF. We used the same protocol that Dr. Leitman described earlier today. The dexamethasone was an 8 mg dose 12 hours before the collection. G-CSF was 5 mcg per kilogram subcutaneously about 18 hours before the collection.

And then when we gave both, it was 8 mg of dexamethasone and 5 mcg of G-CSF per kilogram. Only at the NIH, but this is a double-blind study. So, yes, we did have placebo tablets or dexamethasone, and we did either give G-CSF or a placebo injection.

So we did look at symptoms and this was a nice way to try and sort out the effects of dexamethasone from G-CSF. We plan to enroll 24 donors. I have enrolled 10 so far and I am going to show you the data on 6. Each donor would be randomized to one arm and then we would study their blood chemistries for several weeks and then they would come back six weeks later to be enrolled a second time and a third time. So all three donors got each -- all of the donors got each of the three mobilization regimens and had granulocytes collected. What we measured was symptoms, blood counts, and blood chemistries. I am going to focus mostly on the blood count and chemistry data. We analyzed the donors prior to mobilization, pre and post-collection, and then one and two days after the collection, and then once per week weekly for five weeks.

This is a summary of the platelet counts in the donors. First of all, as you would expect in all three donor groups, the platelet counts fell

after the collection. These counts are premobilization. And as expected, the counts fell about 20 to 30 percent. What we have seen in peripheral blood stem cell donors is that the platelet counts remain low or at about post-collection levels for almost a week. We didn't see that in any of these groups. In the dexamethasone donors, the counts started coming up by day 4. And then about a week after collection, the counts were back into the normal range. We did see a slight overshoot in the counts after two weeks. And by three and four weeks, the counts were back in the normal range. The counts were almost the same in the people who got G-CSF. Again, the counts started to come up -- they were low one day after the collection and started to come up two days and then a week later they were back to normal and two weeks after that they were above normal. A similar effect occurred with G-CSF. So we didn't see any adverse effect of G-CSF on platelet counts. It looks like the recovery of counts is almost identical to donors given dexamethasone.

We also looked at neutrophil counts just to see if there would be any post-collection neutropenia. First of all, as you would expect, the day 1 neutrophil count is about the same. They are

the same group of donors. And as you have seen several times today, the counts are increased to about 20,000 or more with G-CSF and considerably more in people that got G-CSF plus dexamethasone. The day after the collection, the counts were still slightly elevated in the G group and G plus dex, and even slightly higher at day 2. We did not -- the next week and the following week, we really didn't see any difference in granulocyte counts from the pre-G-CSF counts. So based on this data, their granulocyte counts come back to normal baseline quickly, and at least a week afterwards, there would be no reason why a donor could not get another dose of G and donate granulocytes again.

Concerning blood chemistries, it has been well known that when you give G-CSF for three, four, or five days, alkaline phosphatase double or triple. A single dose of G-CSF though does not seem to have a very marked effect on LDH levels. It does increase them though. Baseline levels were at 148, 141, and 146, similar in the three groups. And as you would expect, dexamethasone did not affect the LDH levels after the second day. G-CSF though in both groups did result in a slight increase in LDH levels. And then the day afterward, actually the levels were below baseline. I am not sure why that

was. It may have been due to some dilution following the apheresis.

In contrast to peripheral blood stem cell donors, we did not see any change in alkaline phosphatase. Apparently it takes more than one day of G-CSF to cause changes in alkaline phosphatase.

These data were a little bit surprising in that I did expect the potassium levels to fall when G-CSF was given, and a single dose did result in quite a significant drop in potassium from 4.2 to 3.7, which was back to 4.0 again the day after apheresis. I was surprised though to know that a single dose of dexamethasone can also result in a fallen potassium level. The level went from 4.3 to 3.9. The combined effect of G-CSF and dexamethasone though does not seem to be any different than either drug alone. This change was very transient and by day 3 the potassium levels seemed to be almost back to normal.

Another effect I didn't expect with dexamethasone was a change in albumin levels. With apheresis, donors tend to get some fluids, and I thought it wouldn't be unexpected if they would get a little bit of hemodilution and a fallen albumin. We saw that, but we only saw it in donors that got dexamethasone as opposed to donors that got G-CSF.

So at least by one measure dexamethasone may actually cause more change in blood chemistry and G-CSF doesn't. That wasn't the only parameter that changed slightly with dexamethasone and not G-CSF. Again, albumin went up very slightly. Although these numbers might be statistically significant, I don't think they are going to make any difference for donors. Phosphorus did drop for all three donor groups, but again transiently.

This kind of summarizes what happens with the chemistries we measured. Sodium bicarb, creatinine, calcium, magnesium, bilirubin, alkaline phosphatase and SGPT, SGOT, and GGTs were unchanged. As I pointed out, all donors in all three groups had decreases in phosphorus and potassium. Donors in all groups had a very slight change in cholesterol and triglycerides. Triglycerides might be related to fasting before apheresis. I am not sure on the mechanism of change in cholesterol in the groups. Dexamethasone, as I showed, had a slight decrease in albumin. And then again we saw the data on the bilirubin and the LDH. Chloride was slightly increased in the dexamethasone group and uric acid had a very slight increase in all three groups. The bottom line is I don't think any of these changes are really very significant, and I think that based

on these findings it is going to be safe from a blood count and chemistry point of view to give G-CSF once weekly.

So actually I was quite surprised. This data suggests that in some ways dexamethasone might actually be more potentially harmful to donors than G-CSF. So in summary of this part of the data, the mobilization of granulocytes with either G-CSF or dexamethasone is associated with mild changes in blood chemistries. These blood chemistry changes and cell counts did return to baseline promptly and it may be safe for donors to donate mobilized granulocytes at one-week intervals.

I would like to continue on with the rest of what I did with this study. On this same study, we did collect granulocyte concentrates and we did want to see how well these concentrates stored. The issues I think a lot of people have already mentioned today, though, that granulocyte standards limit storage to 24 hours. And as we have heard many times, G-CSF inhibits apoptosis. It may be possible to store these mobilized granulocytes more than 24 hours and still maintain viability and function. We did have a concern that high cell concentrations in the products that we were

collecting could counter the effects of G-CSF and actually diminish viability.

Again, the study was exactly the same. the same mobilization. We collected the products with a CS3000 blood cell separator and 7 liters of blood were processed. With the CS3000, our volumes tend to be a little bit less than with the products collected in Seattle with the COBE. I will show you later that the products had about 225 ml of plasma. We stored the products at 48 hours, and for this study we measured cell counts, pH, and we also looked at viability. My lab isn't geared up to measure -- not yet anyway to measure a large number of granulocyte functions, but as long as we had these products, we thought we would get some data.

This slide summarizes the first 18 products we collected. Again, it is very similar to what Dr. Leitman has shown earlier today. The volume of these products are all about the same, 230 ml. The white cell concentration though is extremely high in the products. For the dex products, it was 144 times 10^9 cells per liter. It went up to 204 for the G-CSF and 332 for the G plus dex products. I think Tom Lane showed some data where his highest concentration was 80 times 10^9 cells per liter. So this is at least double that

concentration and this is four times that concentration. For a lymphocyte culture, we would usually go with 1 or 2 times 10^9 cells per liter. The composition of the products, again, was similar to reported before. It was 66, 76, and 84 percent granulocytes. And these were the total numbers of cells present, 2.4 times 10^{10} cells and 3.7 times 10^{10} and 6.5. So these are the dose of cells that you have heard many times that people are transfusing practically these days.

The first thing we looked at again was actual counts on the products, either immediately after collection, day 1 or day 2. And as you have seen before, the counts stayed very steady from the first day of storage, at least the dexamethasone products did. We saw a very slight fall-off in counts, less than 10 percent after the second day. Again, the counts were well maintained in both the G and the G plus dex products. So at least by a gross measure by counts, there wasn't much change in the number of cells present.

We looked at cell viability using a 7AAD stain. The cells were almost 99 percent viable the first day, maybe 98 percent viable the second day. We did -- maybe the fall-off went to 90 to 95 percent the second day, but really it really didn't

change much. What we were surprised about though was the pH of these products. As Dr. Lane showed earlier, the pH was 7.1 to start with. I think in his most concentrated products, he had a similar change in pH at 6.3 the first day and then we went to two days and it was 6.1. That wasn't probably too detrimental. But when you go over to the other products, the very concentrated products collected from donors given dex plus G, right immediately after collection the cells were slightly acidic and a day later the pH was 5.5 and stayed that way the second day.

The G stimulated products weren't much better. They were 6.2 pH after day one and 5.8. What was remarkable about these is we saw one or two of these six products have a little bit of hemolysis of red cells by the first day and almost all of them had some hemolysis of red cells present after two days. So even though these cells might look viable, just based on the pH and the presence of hemolysis, I wouldn't transfuse cells with the hemolysis present.

So we think something needs to be done to try and maintain the viability a little better of these cells if we are going to store them. The first thing we thought of is well maybe it has to do

with concentration. How far out can we dilute these granulocyte concentrates in order to maintain the pH. This is a summary of preliminary results with diluting four concentrates out with autologous plasma. What we did was we allocated the concentrates into teflon bags and added autologous plasma and did a 1 to 2, 1 to 4, 1 to 8, and 1 to 16 dilution. I had one product that was -- I lumped the data. One product was collected after dex mobilization and one after G and two after G plus dex. And what we found was that if we diluted the product out 1 to 8 or 1 to 16, we began to have a normal pH and those pH's were maintained in that normal range even after two days. So it looks like diluting a product out just two or four-fold won't be adequate, but somewhere around 8 to 16 fold might maintain the pH. Again, these products were 230 ml to begin with and we can't collect 2 liters of autologous plasma. We are going to try some various additives to see if that will help maintain storage. It may be -- we heard other ideas today. Maybe adding bicarbonate might be another way to go without diluting out the concentrate.

I do agree these are preliminary studies and we do plan to start to measure chemotactic

activity on some of these stored cells to see if there is fall-off in chemotaxis function.

So concluding this abstract, to optimize granulocyte storage, we believe they should be diluted 6 to 18 fold, especially G and dexamethasone mobilized concentrates, or at least some other additive should be added to help maintain the pH. We will probably need clinical grade diluents or additives to maintain the pH. I would like to thank the people that helped me with these studies. Dr. Leitman and members of her apheresis unit, Yu Ying You, Janice Carr, Hatian Chung, who started some of the assays in the laboratory with me, and Dr. Tom Lightfoot, who will be continuing some of these storage studies. Thank you.

CHAIRPERSON HARVATH: Are there any questions for Dr. Stroncek before we start? Maybe what we will do is we will hold the question until the next abstract and we will do it up here. Sorry, because that microphone apparently is not working right now. Now we will try the overhead projector once again. I think we have a new projector.

DR. DIAZ: So this is the last talk of the day. It is Friday. So I promise there are only 14 slides. You only have to be awake for 7 of

them. So I think we can get through this very quickly.

Diaz Law Number 2 of presentations. When someone from a company is presenting something, its credibility is inversely proportional to how slick the presentation is. So in order to aid my credibility, you will see that I have got low-tech black and white, and in the very first slide I have introduced a spelling mistake. Any other spelling mistakes you spot from now on will be due to my aberrant education on a small island just off the coast of Europe.

At Idon, our expertise and our interest is actually in apoptosis. So the neutrophil is really just a by-product of what we have been looking at. And one of the things that we are very interested in is in the caspase dependent apoptosis and cell death, caspases being the enzymes that are involved with the end stage of the death of cells.

Very quickly, the neutrophils obviously have short circulating half-lives in the body. They also seem to lose function and dye when stored in ex vivo, such as in the leukopheresis pack. We know that death in circulating neutrophils is apoptotic. We can only surmise that perhaps the death that we are seeing in the leukopheresis packs is also an

apoptotic cell death. Therefore, what we have been trying to see is if we can interfere with the normal progress of neutrophil death by using inhibitors of apoptosis.

So the two inhibitors that I want to look at very quickly is one which is a generic inhibitor of caspase, I think it is mostly the IL1 beta converting enzyme, which is Z VAD fmk that has been used a lot in different apoptotic research. This is one of our own molecules made by one of our chemists, a highly experienced and wonderful chemist. He is my boss, by the way. Which is the azile dipeptide fmk, indole fmk.

So the assays we have been looking at we set up in order to look at the neutrophils and where we are really inhibiting some neutrophil apoptosis is we looked at oxidative burst assays using zymosan. The reason why we use zymosan is because probably it is more physiologically relevant than something like PMA. It has been shown that cells that are heavily into apoptosis can still have some sort of burst with PMA. And we have been looking at viability assays through flow cytometries like hypodiploidy assay, which is an assay that looks at how intact the neutrophil nuclei are. And the other one is annexin V labeling.

Basically the phosphotidylserine flips out from the inside part of the cell to the outside part of the bilipid layer when the cell goes into apoptosis before it disintegrates. The other thing we have been looking at is CD16. CD16, as Conrad Liles mentioned before, is the FC gamma 3 receptor. It has been shown in several studies that disappearance of the CD16 or expression of CD16 in neutrophils seems to correlate with onset of apoptosis.

So the series of slides I am going to show you are all from isolated human neutrophils from normal donors. So this is not from apheresis packs. This is actually from isolated neutrophils. And what we see is that if we look at the time course of the onset of apoptosis, you can see here that annexin V without treatment, we actually see very quick expression of annexin V labeling with phosphotidylserine on the outside after 24 hours. By 48 hours, just about every cell has gone. When we treat the cell with 1965, this is our sort of shorthand for that azile indole dipeptide, you can see that right after 96 hours we have preservation.

We see similar preservation, although it is not quite as spectacular, when you look at CD16 expression. So again, by 24 hours you have a huge

diminution of the CD16 being expressed in the cell surface of isolated neutrophils. But when you have -- when you incubate these cells in the presence of 1965, you actually see that there is preservation right out to 96 hours where still over 60 percent of the cells are expressing. Just to show you that these numbers aren't made up, we have some pretty colored slides here. You can see here that this is the untreated control at time zero. You can see here that there is a nice population in the facts analysis of a granulocyte population. It is expressing a nice homogenous CD16 population, and it is not labeling with annexin V. With time, 24, 48, 72, and 96, you can see there is a very quick drop off of CD16 and very quick expression of annexin V labeling on the outside, and you can see that here as well in the double staining experiments. Can you see it at the back? With the 1965, you see that you do get preservation. It is not just a figment of my graphs. You can actually see that there is quite good preservation here. Although of course you are starting to see the break-up of the CD16 signal here. Of course these are all surrogate endpoints and we wanted to look also as well at surface markers to see what do the nuclei of these cells look like. So we did the hypodiploidy

assay. This is basically an assay where you, after certain time points, you allow PI or propidium iodine to enter into the cell and label the DNA of the cell and then what you are doing is you do flow cytometry to look at the cells or the nuclei is still attached as opposed to a nuclei that is chopped up. You can see that out to this time point, the 1965 protects the integrity of the nuclei. The fmk has some slide protection, but it is not that great, and you can see how this falls off when it is untreated.

Perhaps more important is what is happening to the actual functional part of the cell. So we -- this is the oxidative burst, what happens in the oxidative burst as I say in response to opsonized zymosan. I think it is probably the most relevant since in order for that to form a response, you have got to have receptors being expressed -- SC receptors being expressed on the outside to capture the cell and to capture the opsonized zymosan. The cell has to have the ability to restructure its cytoskeletan and take in phagocytose and then it has to have a response. So I think it is a very good way of looking at the viability of a cell. You can see that the ability to burst, isolated neutrophils fall very, very rapidly after 24 hours and Z-VAD-fmk

has very little protective activity. The 1965 has protective activity out to 48 hours, which is actually quite good. By the way, I am just showing you -- we have a lot more data than this, but obviously we just wanted to show you representative samples. This is actually at 50 micromolar.

The IC50 of 1965 for 48 hours is about 10 micromolars. So at 10 micromolar, you are still getting 50 percent protection. There are other compounds that seem to be working even better. The 1965 is obviously one that we can show you the structure of.

So not knowing very much about leukopheresis or really about neutrophils, we decided to do something really naive and just put some of these compounds into an apheresis bag thinking we are on to a winner here. If it works on the isolated neutrophils the way that we are mistreating them, it is bound to work in the bag. We set up a series of assays and in particular set up a series of flow cytometry assays looking at these different markers, just so that we could make sure that what we are looking at are neutrophils. So in each case we are labeling with CD3 to identify lymphocytes and CD14 to identify monocytes, although CD14 is expressed in neutrophils, it is actually the

percentage of expression is a lot, lot lower than on monocytes. CD66B is the expression of neutrophils. That is a marker specific for neutrophils. And then obviously this looking for platelets.

In terms of functional markers of cells, we looked at CD16 and CD16B. Really, they are both the same marker. The only difference is that CD16B is a isoform of the CD16. It is actually specific for neutrophils. So that is what mainly we are going to be talking about, CD32. And then most importantly also is CD62L, that is L-Selectin. What we have seen is that when a cell is activated, it loses its L-Selectin.

So we did a whole series of experiments. Rather than go through all that data, we hit several problems of inconsistency, which is sometimes we could get some of our compounds to work and sometimes not. We hit a whole series of problems. And I think a lot of it were also as we went along what we found was that we had other issues other than just stopping apoptosis, which is a lot of these cells were actually not dying of apoptosis I think in the first place. But problems that obviously you are all aware of, but we weren't aware until very recently, which is problems of actual mechanical storage of the samples. Conrad Liles and

other people have obviously gone into this. These are things that we started to sort out on our own.

But really the problem was that we couldn't get through the very first hurdle, which was this. We were actually collaborating with our local blood bank, who I don't think really have -- I am not sure how much experience they have on granulocyte apheresis products. But this is the first hurdle we hit and why we can't give you a straight answer at the moment as to whether these compounds work. This is at time zero. We actually went through -- I think these end up being 11 different leukopheresis packs tested on these days with these different treatments. Unfortunately, our local blood bank also didn't have a G-CSF protocol that we could use.

So these are the different treatments and these are the different dates and here is the problem. If you look right off you can see there is a huge variation from week to week on the cell count that they are actually able to give us. And then even more so the neutrophil count. So the total cell count and the percentage of neutrophils collected in each time point is totally different.

This was a short working day. I am not quite sure how that happened but on the 11th of

February we actually got a sample which had no neutrophils in it whatsoever. The other problem -- even on the days where we actually had cells and even better the days we had cells in the neutrophils, we found that looking at CD62L there is a huge variation in the percentage of cells that are actually labeling with CD62L, which means the state of activation of those cells is totally different. We haven't had a chance to look into this, but I am sure that the state of activation of the cells varies or influences dramatically how the cell will survive and whether the cell is going to go into apoptosis or not.

Lastly, the viability of the cells -- well, at that particular time, there is not that much difference. But I am not quite sure how important that is or how much it is going to vary. Really it is the fact that the number of cells we are collecting and the site of activation of the cells varied so much that we can't really go any further with that particular collaboration.

So we were hoping to come here so that we could get answers like how consistent and we have been pleased with some of the responses here which is how consistent can we expect the neutrophil apheresis product to be. It is obvious that it can

be a lot more consistent. And then also what state can we actually expect them to be by the time we get them.

I think the other thing that will be very important to us is I think it is going to be very difficult for us to do anything with apoptosis inhibitors until we have a system that people agree on of the best way to store the cells and then we can go on from there. Because otherwise, we are going to be trying to interrupt a death process that is more than likely not going to be apoptosis but some mechanical death due to pH, et cetera.

So what next? As I say, this is basically summarizing what I just said. How representative is our source? It looks like it is not very representative of what other people are getting. Certainly not -- we have seen some of the data in terms of consistency historically that Susan Leitman has shown us and that is amazingly different and encouraging.

Very lastly, just a slide of who actually really did the work. The work was actually done by Theresa, Steve, and Shannon in terms of the science and Karent Valentino and David Higgins, who collected a lot of the data and who are our clinical development people and got us in touch with

everyone. I sort of mainly drank coffee, discussed UNC basketball and provided amusing anecdotes. Thank you.

CHAIRPERSON HARVATH: Okay. Dr. Ambruso?

DR. AMBRUSO: I guess this microphone is on. Dave, what kind of bags did you use to collect your samples in?

DR. STRONCEK: We collected them in originally the bags that come with the kit and then we transferred. We tried in larger bags, some live cell bags, and teflon bags. Live cell is a Baxter bag that is supposed to be more breathable, and it didn't seem to make much difference with storage. We also tried teflon bags because we wanted the smaller bags, some with about a 30 or 40 ml capacity so we could aliquot these products and try some different storage conditions. What was available was from a local company here that will manufacture teflon bags in about any size we wanted. So that is why we picked those.

DR. AMBRUSO: These are not appreciably or don't allow enough gas exchange perhaps like platelets?

DR. STRONCEK: Well, the teflon bags are supposed to be extremely breathable. They are supposed to be better than some of the other bags.

DR. AMBRUSO: You didn't see -- I mean one of the ways to approach this might be if you allowed, as with platelet storage, if you alloed CO_2 to diffuse out. Maybe that might help with the pH.

DR. STRONCEK: Yes. That would be one of the things to try.

AUDIENCE MEMBER: Hi, Dave. I don't know much about neutrophils, but if given a carbon source, will they take one that would go into the Krebs cycle rather than glycolysis?

DR. STRONCEK: I don't know. Do you mean what if we incubated them in a CO_2 incubator at room temperature?

DR. TORLINI: Or acetate. People are looking at platelets for acetate. So you don't make an acid by-product.

DR. STRONCEK: That would probably be worth a try rather than trying to add or dilute out the products.

AUDIENCE MEMBER: Yes. That is what I was thinking.

CHAIRPERSON HARVATH: Okay. I would like to thank everyone who participated in this

conference and all of those of you who have stayed here to the closing moments of the conference. I hope our colleagues at NIH who were here to hear about the areas that are in need of research support in this area heard what all of you had to say. I know that one of the reasons for having this meeting transcribed was so that we could have a record of the current thinking in this area. And also we look forward to, I think, continuing the dialogue and looking at the progress in this area, particularly those of you who have already discussed your intentions to pursue a multi-center type of study design to look at these basic issues. I don't know if Dr. Snyder is still in the audience. Is he here? No. Okay. I would like to also thank him for his comments and input during the meeting as well and some of the suggestions that he had. It has been a long couple of days. We have heard a lot and I want to again thank all of you on behalf of the organizing committee and let you enjoy what is left of your Friday afternoon.

Thank you.

(Whereupon, at 3:41 p.m., the workshop was concluded.)