(Applause.)

DR. SALOMON: The tradition has not been to clap for presentations, but I think that that tradition isn't appropriate when someone is presenting their data.

DR. SHAPIRO: Thank you.

DR. SALOMON: Data takes precedence, nicely done.

Phil?

DR. NOGUCHI: Like Jay, I'll be very brief.

I direct the Division of Cellular and Gene Therapies where you have, over the last, quite a number of occasions been reviewing problems that we have in terms of product development related to cell therapies, gene therapies and the like.

I'd like to present here now, Dr. Tom Eggerman who is a member of the Laboratory of Molecular Tumor Biology.

DR. EGGERMAN: Thank you, Phil. Good afternoon. For my site visit review I will be presenting a talk about the identification and characterization of binding proteins for high density lipoproteins, usually referred to for short HDL. Before proceeding to the data I want to give some

background information regarding the importance of HDL, describe what HDL is, what its role is in lipid metabolism and then review some aspects about known HDL binding proteins.

Heart disease is the number one cause of death in this country and atherosclerosis is a major contributor to heart disease. Several risk factors have been identified for atherosclerosis, among them are low levels of high density lipoproteins and this data is from the Framingham Heart Study and in this figure, the relative risk of developing coronary artery disease in men is shown to decrease as the levels of HDL increase.

In contrast, levels of low density lipoprotein cholesterol, so-called bad cholesterol, LDL cholesterol, there's an increased relative risk of coronary artery disease.

With the initial national cholesterol education efforts, the emphasis was on ways of reducing total and LDL cholesterol. More recently, the emphasis has expanded, recognizing the importance of HDL cholesterol, in fact, most major drug companies are now developing drugs, specifically targeting for elevation of HDL cholesterol.

Clinical studies such as the VA HDL

Intervention Trial are suggesting that raising HDL cholesterol levels is also beneficial and for this reason because the therapeutic interventions are so limited, it's critical and HDL metabolism be more fully understood and so that the best therapeutic interventions can be developed.

The characteristics of high density lipoproteins are shown in this figure. These lipoproteins exist as articles with a diameter of 5 to 12 nanometers with a lipid core and hydrophilic surface of principally protein and phospholipid. Their size is 1.7 to 3.6 times 10^5 daltons and the lipid consists of 5 to 10 percent of triglycerides, 15 to 25 percent cholesterol and 20 to 30 percent of phospholipids. The remaining 35 to 60 percent is protein.

Next. There are several proteins called apolipoproteins which can be found associated with HDL and provide structural integrity to these particles. The two major ones are Apolipoprotein A-I or ApoA-I for short with a mass of 28,000 daltons and Apolipoprotein A-II which exists as a dimer and has a mass of 17,000 daltons. Since these proteins exist on the surface of lipid particles, it is thought that they are also ligands involved in mediating many of

the HDL effects.

Next slide, please. HDL is known to have at least two functions, the well known involvement in lipid metabolism, especially cholesterol transport and the lesser known function of acting as a carrier for lipid soluble materials. An example of this is lipopolysaccharaides or LPS. I will be presenting data demonstrating that these two functions can potentially overlap.

A synopsis of the metabolism of HDL is shown in this figure. The primary function of HDL is to mediate cholesterol movement from the periphery to the liver, the so-called reverse cholesterol transport. The initial source of HDL called nascent HDL is from the liver and intestine where newly synthesized ApoA-I is released with only a small amount of lipid.

In the presence of peripheral cells, nascent HDL is able to accumulate free cholesterol from a plasma membrane. A candidate binding protein is supposed to be responsible for this activity is called ABCA-I or ATP binding cassette I protein which mediates cholesterol translocation from intracellular stores to the plasma membrane. A defect in this protein causes Tangiers Disease which is associated

with very low levels of HDL cholesterol. It is thought that there could be other HDL binding sites on the surface that could have other functions such as signalling. As I had mentioned previously, another function for HDL is carrying the lipid soluble molecules such as LPS and this is demonstrated here in this slide. SRB-I or scavenger receptor B-I is found on the liver as well as steroidogenic tissues including the adrenal gland, ovaries and testes and takes up cholesterol estrorich particles including HDL. As a scavenger receptor, it can recognize multiple ligands and also it will take up multiple types of lipids. Other HDL receptors have been proposed and may exist on the liver or potentially other cells that are involved in the uptake of HDL particles.

The identified binding proteins that I showed on the previous slide, the ABCA-I and the SRB-I have KDs that correspond to a relatively affinity site. From the literature, including our own lab, statute analysis of HDL binding indicates that there is a higher affinity site or sites so as yet to be identified, HDL binding proteins must exist to correspond to these higher affinity binding sites.

There also have been several observations

suggesting additional HDL binding proteins. These include what's called the retroendocytosis of HDL particles where HDL particles are taken up by the cell or liver and then resecreted. The uptake of HDL remnant particles after metabolism, signal transduction as I mentioned earlier and also LPS uptake.

Next slide. I will now begin presenting some of our data on novel HDL binding proteins that we have identified by modifying a Western blot like assay called the Ligand blot. We've been able to identify three proteins of 100, 95 and 55 kilodaltons that all bind HDL. We have identified and characterized these proteins, particularly to determine if they correspond to already known HDL binding proteins. Because of limited time I'll not be able to review all this work as I did in the October site visit. I will briefly describe the results for the 100 kilodalton protein and for the 95 kilodalton protein and then present some of the data that we have on the 55 kilodalton protein.

The 100 kilodalton protein after purification and microprotein sequencing turned out to be glucose regulatory protein 94 which had been recently identified as an HDL binding protein in 1999

by another group. The 95 kilodalton protein was characterized and the results will be coming out shortly in the Journal of Biochemistry and we're still in the process of purifying and attempting to identify this protein.

Regarding the 55 kilodalton protein, we purified the 55 kilodalton protein from plasma membranes. On this slide the results are shown after the final purification step of two dimensional electrophoresis. On the left panel with Kalassy stain, we observed a triplet, appropriate size, and in the right panel using this Ligand blot and the ligand that we used with aprolipoprotein A-II, one of the proteins on HDL, we also observed the triplet. After HPLC purification, the cutout band was then microprotein sequenced. Six piques were found to be 100 percent homologous with heat shock protein 60 of Hsp60. In addition, mass spectroscopy fragmentation analysis was consistent with Hsp60.

Next slide, please. Hsp60 or heat shock protein 60 belongs to a family of heat shock proteins which are inducible or constitutively expressed by prokaryotic and eukaryotic cells. It's also called the mitochondria matrix molecular chaperon, is involved in the appropriate folding of proteins, about

10 to 20 percent of this protein is found in the cell surface.

Multiple diseases are associated with heat shock protein 60 antibodies such as lupus erythematosus and other autoimmune disease and even Type I diabetes. These diseases are usually associated with accelerated atherosclerosis. Antibodies to heat shock protein 60 are positively correlated with the development of atherosclerosis in patients and in animal studies, exposure to microbial heat shock protein 60 is associated with a development of atherosclerosis.

Next slide, please. In this slide we are demonstrating the specificity of the aprolipoprotein, that's one of the proteins on HDL binding to heat shock protein 60 and what we have is the binding in this assay to the 55 kilodalton protein as evaluated by Ligand blot and would show that in the presence of increasing amounts of heat shock protein antibody, called LK-I, that we're able to see decreasing amounts of ApoA-II binding.

Next slide, please. It's known that heat shock treatment of cells result in increased heat shock protein 60 expression on the cell surface. In this slide, we have heat shock treated fibroblasts for

30 minutes at 42 degrees and then evaluated, labeled HDL binding to the cells and comparing specific HDL binding which is shown as the third result in each one of these pictures.

Heat shock treated cells had almost three times as much HDL binding as the control cells suggesting that increased heat shock protein 60 expression is associated with the increased HDL binding.

Next slide, please. A significant question is whether or not the binding of HDL or APoA-II is truly physiologic since heat shock protein 60 is known to bind multiple proteins as part of its mitochondrial matrix chaperon function. When apolipoprotein A-I, again, this is another protein that's found on high density lipoproteins is added to multiple cell types, including fibroblasts, it's been demonstrated to increase cholesterol release from these cells and this is called cholesterol efflux.

We have evaluated ApoA-I induced cholesterol efflux from human fibroblasts in the presence and absence of these three antibodies to heat shock protein 60 that are each targeted to different epitopes of heat shock protein 60. All three of them indicate that they're able to decrease cholesterol

efflux, suggesting that heat shock protein 60 indeed does have a physiological HDL metabolism.

Next slide, please. Further work to evaluate the significance of heat shock protein 60 in atherosclerosis includes a collaboration with Steve Epstein who was formerly at the NIH and now is at the Washington Hospital Center and he published a paper earlier this year entitled "Antibodies to Human Heat Shock Protein 60 Associated with the Presence and Severity of Coronary Artery Disease, Evidence for an autoimmune Component of Atherogenesis." We intended to use this antisera from these same patients to determine if heat shock protein 60 antisera blocks Apolipoprotein binding to heat shock protein 60 and if it also will block cholesterol efflux and then correlate these effects with the presence and severity of coronary artery disease.

As I indicated in the beginning of my talk, HDL has an additional function as a carrier of lipid soluble materials. This is figure, LPS which originates from the cell wall of gram negative bacteria demonstrates alternative pathways which are catalyzed by an enzyme called LPS binding protein or LBP. This enzyme has significant homology to various enzymes in lipid metabolism highlighting the

similarities between LPS metabolism and lipid metabolism. The alternative pathways shown on this slide include being bound by CD-14 resulting cellular signal transduction, citokine release through well characterized pathways and the possibility of developing endotoxic shock.

Alternative LPS can bind to HDL which appears to be protective and results in neutralization and perhaps also in being directed to a degradation pathway.

Next slide, please. The structure of Lipopolysaccharide is shown on this slide. The major structural aspects are the fatty acid portion at the bottom, the polysaccharide portion which is in the upper part and the phosphate groups.

These components are also found in different combinations in the normal constituents of lipid particles such as phospholipids, triglycerides and cholesterolestese. LPS can also be referred to as endotoxin and is associated with a development of septic shock as I said earlier. Multiple FDA investigative new drug applications have been evaluated, attempting to treat this frequently fatal condition.

The absence of endotoxin in a product is

an important FDA criteria for allowing its use in patients. And recently, the presence of bacteria such as chlamydia have been identified in atherosclerotic plaques and the release of LPS from these plaques is now being suggested as a potential pro-atherogenic factor.

In looking at the literature, a 20-year-old study published in JCI, evaluated the uptake of labeled Lipopolysaccharide that had been associated with HDL. They observed in control animals that the highest tissue density of uptake was in the adrenal followed by the ovary and the liver. These three tissues correspond to the same location where the HDL binding protein SR-BI is localized. When animals were pre-treated with dexamethasone, a treatment known to significantly downregulate SR-BI, the updated in the adrenal was significantly decreased and almost eliminated.

When animals were pre-treated with ACTH, or adrenocorticotrophic hormone, a treatment known to up regulate SR-BI, the update in the adrenal was increased. This study suggested to us that SR-BI could be involved in LPS tissue updated.

Next slide, please. In this first experiment we evaluated the potential binding of

Lipopolysaccharide to SR-BI by evaluated labeled LPS in SR-BI overexpressing and control cells. And the overexpressing cells that are seen in the closed circles demonstrated a much higher level of binding than the control cells suggesting that SR-BI actually could act as a receptor.

Next. To further evaluate this increased binding observation, we evaluated the uptake of LPS into control and the same SR-BI overexpressing cells. The overexpressing cells in the open squares demonstrated a significantly increased amount of uptake, suggesting that LPS not only binds to SR-BI, but this increased binding is associated with an increased uptake into the cell.

Next slide, please. In this slide, LPS update is evaluated using a fluorescently labeled LPS. This compares SR-BI overexpressing and control cells and one can see that there's a significantly increased amount of fluorescently labeled LPS uptake in the SR-BI overexpressing cells. The location for uptake is consistent with the Golgi apparatus where LPS is normally transported intracellularly.

If LPS binds to SR-BI, one would expect that LPS could compete with HDL and proteins on HDL such as ApolipoproteinA-I. In this figure, the

competition of ^{125}I labeled HDL is determined in competition with LPS and unlabeled HDL. LPS is a very effective competitor. It's in the red open circles and has an IC50 of about 5 micrograms per milliliter. Unlabeled HDL which is in the open squares is a much less good competitor.

Next slide. Using labeled Apoliprotein A-I, we also looked at competition between LPS and ApoA-I and what we saw in this case was that both LPS and ApoA-I had very similar competition curves and the IC-50 for LPS was around 2 micrograms per milliliter.

We have just begun to evaluate the interaction with the other major HDL binding protein ABCA-1 and LPS. In this slide, the two inhibitors of ABCA-1 were used to evaluate LPS uptake. These two inhibitors are sulfobromophthalein and DIDS. Both inhibitors significantly decreased uptake, suggesting that ABCA-1 may also be involved in LPS metabolism.

Next slide, please. Potential model for our results is seen in this slide. SR-BI, as I mentioned earlier is known to be the primary binding site for cholesterol ester uptake. How cholesterol ester is then shuttled to intracellular stores, however, is not really known. Our data suggests that SR-BI may have an additional role as an LPS receptor.

SR-BI's known role as a scavenger receptor that takes up many different lipids from multiple ligands is consistent with this possibility.

In the lower part, I have ABCA-1, a protein that's primarily involved in free cholesterol mobilization from intracellular compartments. Dr. Bryan Brewer's lab has recently demonstrated that ABCA-1 functions as an intracellular shuttle delivering free cholesterol to the cell surface of intracellular stores.

Our initial work observing effect on ABCA-1 on LPS uptake suggests that ABCA-1 may have a role in LPS metabolism and one potential function could be that ABCA-1 acts an intracellular shuttle to carry LPS intracellularly from the cell surface.

As I mentioned earlier, LPS has a significant signal transduction activity that results in cytokine release and the development of endotoxic shock. We next evaluated how LPS affects the expression of HDL binding proteins. In this slide, raw cells which are a mouse macrophage cell line were treated with LPS. Response of both ABCA-1 on the right and SR-BI on the left demonstrate a dramatic reduction of greater than 80 percent in MR and A levels within 24 hours. Of particular note is ABCA-1

where the potency of the LPS effect is such that complete ambition occurs at only two nanograms per milliliter.

Next slide, please. In evaluating response of SR-BI protein to LPS treatment, we used a Western blot analysis for these raw cells. A similar dose response curve was seen as in the previous slide and this case we're looking at the protein instead of the MRNA and again an 80 percent reduction is seen at a concentration of about 2 nanograms per milliliter.

Next slide, please. To further evaluate SR-B1 and the effect of LPS, the activity of SR-B1, that is, cholesterol ester uptake was evaluated in response to LPS. Nearly a complete inhibition is seen of cholesterol ester uptake and these plus the two previous slides demonstrate dramatic reductions of SR-B1 and MRNA protein inactivity in response to LPS.

Next slide, please. Conclusion, we have identified three HDL binding proteins. The 100 kilodalton protein was determined by micro protein sequencing to be the already known HDL binding protein, glucose regulatory protein 94. The 95 kilodalton protein has been characterized. We're still in the process of doing further purification and with the hope of identifying whether this is an

established or new protein.

With the 55 kilodalton protein, we've determined this to be heat shock protein 60 by microprotein sequencing. The potential physiologic involvement of this protein has been demonstrated by heat shock protein 60 antibodies binding, preventing binding of ApoA-II to the protein and inhibiting ApoA-I stimulated cholesterol efflux.

In addition, heat shock treatment which increases heat shock protein 60 expression on cell surface increases HDL binding. The sum of these observations suggests that heat shock protein 60 is a novel HDL binding protein which is important in HDL metabolism that may provide a mechanism to explain the known association between immunity developed against heat shock protein 60 and the development of atherosclerosis.

We also presented data demonstrating an interaction between ABCA-I, one of the two primary well-known HDL binding proteins and LPS. We demonstrated that inhibitors of ABCA-I decreased LPS uptake and that LPS down regulates the expression of ABCA-I. Further studies are needed to define this interaction between ABCA-I and LPS to determine what role ABCA-I has.

Next slide, please. And we've also presented data indicating that there's a significant interaction between the HDL binding protein called SR-BI and LPS. Overexpression of SR-BI results in increased binding and uptake of LPS. LPS competes with both HDL and ApoA-I with binding and LPS downregulates SR-BI MRNA protein and activity.

Next slide, please. I want to acknowledge all those who have helped us in these studies and CBER, from my lab, Alexander Bocharov and Shaobin Zhong, also from the Division of Therapeutic Proteins, Ray Donnelly and Harold Dickensheets. From the NIH, Amy Patterson and her group, including Irina Baranova, Zhigang Chen and Tatiana Vishykanova and from Dr. Brewer's group himself and Alan Remaley.

Thank you very much.

(Applause.)

DR. SALOMON: Thank you very much, Tom. Are there any questions from the committee to either of the presenters?

Well, then what I'd like to do is take a break while we go from a public to a closed session where the presentation of the results of the site review by Dr. Sausville will take place.

Thank you all very much. Thank you to

both the presenters who did a beautiful job and for the rest of you, we'll see you back here in a couple of minutes.

(Whereupon, at 5:20 p.m., the open meeting was adjourned.)

CERTIFICATE

This is to certify that the foregoing transcript

in the matter of: BIOLOGICAL RESPONSE MODIFIERS ADVISORY COMMITTEE

Before: FOOD AND DRUG ADMINISTRATION
CENTER FOR BIOLOGICS EVALUATION
AND RESEARCH

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represents the full and complete proceedings of the aforementioned matter, as reported and reduced to typewriting.

Laurie Rosebach