RECOGNITION OF ENDOTOXIN IN BIOLOGIC SYSTEMS

Organizers: Richard J. Ulevitch and Peter S. Tobias

March 1-7, 1992

Plenary Sessions Page

March 2:
Biosynthesis, Structure and Chemistry of Natural and Synthetic Lipid A 150
Proteins with Lipid A Binding Sites 150

March 3:
Candidates for Lipid A Receptors 152
CD14 and a New Paradigm for LPS Recognition By Cells 153

March 4:
LPS-Induced Cell Stimulation Via CD14 154
Signal Transduction Mechanisms of LPS 155

March 5:
Effector Mechanisms for LPS Action 156

March 6:
New Opportunities for Therapy-I 157
New Opportunities for Therapy-II 158

Late Abstract 158

Poster Sessions

March 2:
LPS and LPS Binding Proteins (CB100-116) 159

March 3:
LPS Receptors and CD14 (CB200-208) 163

March 4:
Cell Activation (CB300-312) 166

March 6:
Therapy (CB400-416) 169

Late Abstract 174

Biosynthesis, Structure and Chemistry of Natural and Synthetic Lipid A

CB 001 LIPID A STRUCTURE: MOLECULAR RELATIONS TO ACTIVITY AND FUNCTION, Ernst Th. Rietschel[1], Lore Brade[1], Helmut Brade[1], Hans-Dieter Flad[1], Harald Loppnow[1], Ulrich Schade[1], Ulrich Seydel[1], Artur J. Ulmer[1], Ulrich Zähringer[1] and Shoichi Kusumoto[2], [1]Forschungsinstitut Borstel, D-2061 Borstel, FRG and [2]Osaka University, Dept. of Chemistry, Osaka 560, Japan.

Lipopolysaccharides (LPS) constitute the O-antigens and endotoxins of Gram-negative bacteria. They are essential for microbial viability and play an important role in the pathogenesis and toxic manifestations of bacterial infection. Chemically, LPS consist of a polysaccharide portion and a lipid component, termed lipid A. The endotoxic activities of LPS are mediated by lipid A. The primary structure of lipid A of various bacterial origin is known. As an example, Escherichia coli lipid A consists of a 1,4'-bisphosphorylated β-D-glucosaminyl-(1-6)-α-D-glucosamine (GlcN II-GlcN I) disaccharide which carries four (R)-3-hydroxytetradecanoic acid residues (at positions 2, 3, 2' and 3'), of which that at 2' is 3-O-acylated by dodecanoic (12:0) and that at 3' by tetradecanoic acid (14:0). The hydroxyl groups in positions 4 and 6' are free, the latter serving, in LPS, as the attachment site of the polysaccharide portion via Kdo.

E.coli type lipid A expresses strong endotoxic activity as determined by the induction of protein mediators (TNF, IL-1 and IL-6) in human peripheral monocytes (MNC). By means of natural and synthetic analogues and lipid A partial structures it was found that endotoxicity is not influenced by replacement of GlcN by GlcN3N and of the glycosidic phosphate by an α-2-phosphonooxyethyl group. In contrast, significantly lower activity is displayed by compounds in which the anomeric configuration of GlcN I is β, in which 14:0 is present in a different location, in which acyl groups have a reduced chain length, and which lack a phosphoryl or one (or more) acyl groups. Thus, precursor Ia (synthetic compound 406), differing from E.coli lipid A by the absence of 12:0 and 14:0, lacks mediator-inducing capacity in MNC. These observations point to an extreme structural specificity of the expression of endotoxicity and suggest that in the interaction of lipid A with monocytes a specific cellular receptor is involved. In accord with this concept we found that compound 406 strongly competes with the specific binding of LPS to monocytes and that it antagonistically inhibits the production of LPS-induced TNF, IL-1 and IL-6.

To elucidate the physical structure which is involved in lipid A bioactivity, theoretical calculations and synchroton small-angle X-ray diffraction measurements were performed. These studies allowed to define models i) of the molecular conformation of lipid A [1] and ii) of its supramolecular arrangement. The latter was found to depend on both intrinsic factors (primary structure) and extrinsic ones (T, Me^{2+} concentration, pH, H_2O content). Under physiological conditions, lipid A (and Re LPS) adopt, in the gel (β) state, cubic (and lamellar) structures, whereas in the liquid-crystalline (α) state, hexagonal (H_{II}) phases predominate. These phase states correlate with biological effects of lipid A.

These and other data lead us to conclude that a structure to display endotoxic activity requires a certain equilibration of hydrophilic (bisphosphorylated β-(1-6)-linked gluco-configurated HexpN[3N] backbone) and hydrophobic (defined number and location of fatty acids having a specific chain length) regions. The arrangement of such regions as present in the unique primary structure of E.coli lipid A results in the formation of a peculiar conformation (endotoxic conformation) and a particular supramolecular organization, which allows the expression of endotoxic activity [2].

[1] Kastowsky et al., Eur. J. Biochem 197 (1991) 707
[2] Rietschel et al., Adv. Exp. Med. Biol. 256 (1990) 81

CB 002 SYNTHESIS AND SOME BIOLOGICAL PROPERTIES OF LIPID A ANALOGS, Peter L. Stütz, Bulusu Murty, Jacques Eustache, Johannes Hildebrandt, Charles Lam, Ekke Liehl, Ingolf Macher, Peter Mayer, and Hermann Vyplel. Sandoz Forschungsinstitut, Brunnerstrasse 59, A-1235 Vienna, Austria.

Synthetic analogs of lipid X, the reducing end of lipid A were converted into the corresponding UDP-derivatives and coupled to a range of other synthetic lipid X analogs with free hydroxyl group at position 6 in the presence of a crude preparation of bacterial lipid A synthase to form disaccharide analogs of the biosynthetic lipid A precursor, compound 405, in rather good yields. These compounds behaved either as immunostimulatory mimetics of bacterial lipopolysaccharide (LPS) or as LPS-antagonists. Structure-activity relationships will be briefly discussed. As next step, acyclic analogs of lipid X or compound 405 were prepared which are, in fact, N,O-acyclated peptides without acylated glucosamines. Superficially, these lipopeptides would not be recognized as lipid A analogs any more. Surprisingly, these compounds show a biological spectrum of activities very similar to LPS suggesting that binding of lipid A itself to receptor sites takes place at its lipophilic site. In many cases, these acyclic lipid A analogs show higher endotoxic side effects than the immunostimulatory monosaccharide lipid A analog SDZ MRL 953 which is in preclinical development. Of the latter compound a brief summary of biological properties evaluated so far will be presented. Emphasis will be laid on its capacity to selectively induce monokines which can cause hematopoietic recovery in myelosuppressed mice. In healthy rhesus monkeys, a single administration of SDZ MRL 953 induces massive release of G-CSF, IL-6, but only slightly increased TNF-α levels. Daily administration for 5 days caused a complete down-regulation of G-CSF but not of IL-6 levels from day 2 on

Proteins with Lipid A Binding Sites

CB 003 HOST PROTECTION AGAINST GRAM-NEGATIVE BACTERIA AND ENDOTOXIN BY THE BACTERICIDAL/PERMEABILITY INCREASING PROTEIN (BPI) OF NEUTROPHILS, Peter Elsbach[1], Jerrold Weiss[1], Chean Eng Ooi[1], Martin Doerfler[1], Chen Shu[1], Lynn Grinna[2], Arnie Horwitz[2], Georgia Theofan[2], and Ada Kung[2], [1]New York University School of Medcine, New York, NY 10016 and [2]XOMA Corporation, Berkeley, CA 94710.

In the array of antimicrobial agents and systems, that are presently recognized in phagocytes, the Bactericidal Permeability Increasing protein (BPI) of PMN stands out because of its cytotoxic specificity for gram-negative bacteria (GNB). This selective action reflects a strong attraction for the lipopolysaccharides (LPS) of the GNB outer envelope. Both the cDNA of this ca. 55 kDa Lys-rich protein, isolated from human, cow and rabbit and the functional properties of the purified proteins show high level conservation. The potencies against GNB of BPI and of its equally active ca.25 kDa N-terminal fragment exceed those of all other isolated PMN proteins and peptides by >10-fold. The C-terminal half lacks antibacterial activity. The fates of GNB ingested by PMN or exposed to isolated BPI/fragment are nearly identical, suggesting strongly that BPI fulfills a major role within the intact PMN. Isolated or recombinant BPI or N-terminal fragment(s), added to whole blood *ex vivo* kill surviving *Escherichia coli* and neutralize the release of TNF in response to the introduction of whole bacteria or isolated LPS. Opal et al. have further shown that holo-BPI protects animals against the lethal effects of administered LPS and GNB. We have shown similar protection with the N-terminal fragment. Thus, BPI and the N-terminal half of the molecule act in host-defense against GNB, both by arresting proliferation of invading bacteria and by blocking LPS-induced destructive host responses.

CB 004 LPS-SENSITIVE COAGULATION CASCADE IN LIMULUS HEMOCYTES, Sadaaki Iwanaga, Tatsushi Muta, Fuminori Tokunaga, and Toshiyuki Miyata, Department of Biology, Faculty of Science, Kyushu University, Fukuoka, Japan.

Horseshoe crab (Tachypleus tridentatus) hemocytes contain a coagulation system highly sensitive to bacterial endotoxin. This system involves at least three intracellular serine protease zymogens, named proclotting enzyme, factor B, and factor C. The LPS-sensitive serine protease zymogen, factor C, is a key enzyme to initiate the coagulation cascade. We have determined the entire amino acid sequence of factor C using recombinant DNA technique. The zymogen factor C consisted of 994 amino acid residues with a calculated molecular mass of 109,648 Da. Most interestingly, factor C has five repeating units ("Sushi" domain or short consensus repeat) of about 60 amino acid residues each, which have been found in many proteins participating in the mammalian complement system. In addition to a typical serine protease domain in the carboxyl-terminal portion, characteristic segments with an epidermal growth factor-like, a C-type lectin-like, a cysteine-rich, and a proline-rich domain were also found, revealing a unique mosaic protein structure. The serine protease domain was most analogous to human thrombin. Furthermore, we identified a transcript possibly derived by alternative splicing of factor C mRNA, which encodes a protein sharing the amino-terminal portion of factor C.

We also studied on the intraction between LPS and factor C to elucidate the LPS-mediated activation. The rate of activation of the zymogen factor C was higyly dependent on the concentration of LPS and on temperature, and the curve of amount of LPS versus activation showed saturation at 37°C. Moreover, a high-molecular-mass complex formed between factor C and LPS was found in a gel-filtration experiment on a Sepharose 4B column. This complex formation was also confirmed by double diffusion analysis on agarose plates. Triton X-100, which destroys LPS micelles, strongly inhibited the LPS-mediated activation of factor C but not activated factor $\overline{C}$. These results indicate that the binding of factor C with LPS is requred for its activation and that only LPS-associated factor C generated the active factor $\overline{C}$. On the other hand, the LPS-mediated activtion of factor C was strongly inhibited by the S-alkylated heavy chain containing a Cys-rich-, a lectin-like and four "Sushi"-domains. In contrast, the S-alkylated factor C -light chain containing a "Sushi"-, a proline-rich- and a serine protease domains did not show any inhibitory effect on the activation of factpr C, suggesting that the heavy chain located in the NH_2-terminal portion of factor C contains an LPS-binding region.

CB 005 ACYLOXYACYL HYDROLASE, A LEUKOCYTE ENZYME THAT PREFERENTIALLY HYDROLYZES SATURATED (OR SHORT) ACYL CHAINS FROM BACTERIAL LIPOPOLYSACCHARIDES AND GLYCEROLIPIDS. Robert S. Munford and Jay P. Hunter. Departments of Internal Medicine and Microbiology, University of Texas Southwestern Medical Center, Dallas, TX.

Acyloxyacyl hydrolase (AOAH) was identified in leukocytes and purified from HL-60 cells by following its ability to deacylate bacterial lipopolysaccharides (LPS). Although enzymatic deacylation reduces the potency of purified LPS in several bioassays, the biological roles of AOAH are not known. To obtain further information about the possible functions of the enzyme in cells, we measured the activities of purified AOAH toward several non-LPS substrates in vitro and tested its ability to act as an acyltransferase.

We found that AOAH can act as a phospholipase, lysophospholipase, and diglyceride lipase. While the enzyme preferentially removed palmitate or stearate from the sn-1 position of phospholipid or diacylglycerol substrates that have unsaturated acyl chains in the sn-2 position, the enzyme cleaved both palmitates from sn 1,2 dipalmitoylphosphatidylcholine and dipalmitoylglycerol. An identical pattern of substrate deacylation was found using recombinant AOAH. This apparent preference for removing saturated (or shorter) acyl chains from glycerolipids is consistent with its ability to cleave laurate more rapidly than palmitoleate from lipopolysaccharide (1). The ability of the enzyme to deacylate LPS in vitro was approximately 10 times greater than its ability to act on the other substrates. AOAH also catalyzed the transfer of acyl chains from LPS and phospholipid to monoacylglycerol to form diacylglycerol.

AOAH has two disulfide-linked subunits. The larger peptide, M_r = 50,000, has a typical lipase consensus sequence, whereas the small subunit (M_r = 6,000 - 10,000) has sequence similarity with the sphingolipid activator proteins (saposins) and surfactant protein B (2). Experiments are currently underway to test the hypothesis that the large subunit contains the catalytic site of the enzyme and the small subunit confers its ability to attack lipopolysaccharides. The novel structure of the enzyme may account for its unique substrate range.

The demonstration that AOAH has phospholipase, lysophospholipase, diacylglycerol lipase, and acyltransferase activities in vitro suggests that the enzyme may have roles in addition to LPS deacylation (detoxification) in phagocytic cells. For example, its presence in alveolar macrophages and its ability to deacylate dipalmitoylphosphatidylcholine, the principal phospholipid in surfactant, suggest a possible role in surfactant turnover. Another role is suggested by its ability to act as an acyltransferase: since LPS molecules that lack secondary (acyloxyacyl-linked) acyl chains are not stimulatory to human cells, perhaps transfer of these fatty acids from LPS to acceptor lipids or proteins is a critical step in the transmission of the toxic LPS signal within certain cells. We are using radiolabeled LPS to test this hypothesis in human macrophages.

(1) A.L. Erwin and R.S. Munford, J. Biol. Chem. 1990; 265:16444-16449.

(2) F. S. Hagen et al., Biochemistry 1991;30:8415-8423.

CB 006 LIPOPOLYSACCHARIDE BINDING PROTEIN. Peter S. Tobias, Katrin Soldau, Loren Hatlen, Ralf Schumann, Greg Einhorn, John Mathison, and Richard Ulevitch, The Scripps Research Institute, La Jolla, CA.

Lipopolysaccharide binding protein (LBP) is a 60 kDa serum synthesized in hepatocytes as a 50 kDa precursor and secreted after glycosylation. LBP is an acute phase reactant. In humans, LBP rises from 7.2 +/- 4.0 ug/ml (n=44) in the sera of normal subjects to 220 +/- 100 ug/ml (n=21) in acute phase subjects. Sequencing of cDNA clones for rabbit LBP (R-LBP) and human LBP (H-LBP) reveals two very similar mature proteins of 456 aa preceded by signal peptides of 26 or 25 residues, respectively. R-LBP and H-LBP have, respectively, three and five potential glycosylation sites, all in the carboxyl half of the molecule. There is no obvious lipid binding domain revealed by hydropathy plots. Recombinant H-LBP and R-LBP, expressed in baculovirus and CHO cells, respectively, bind LPS with essentially the same dose dependencies as the native LBPs. Thus variations in carbohydrate structure are unlikely to be significant for LPS binding activity. Among LPS binding proteins, LBP shows significant similarity only to BPI, an LPS binding protein of neutrophil granules. Smooth and rough form LPS do not differ dramatically in their binding to LBP and have Kd's in the nanomolar range. The KDO sugars derived from Re595 LPS do not inhibit the binding of Re595 LPS to LBP although synthetic lipid A does, thus the primary interaction of LBP with LPS appears to be with the lipid A moiety of LPS. The LPS dependent activation of cytokine production by macrophages, monocytes (MO) and monocytic cell lines has been shown to be markedkly enhanced by complexation of LPS with LBP. Effective doses of LPS are 100-1000 fold lower and responses, for example TNF production from rabbit PEM, are more rapid in the presence of LBP at levels as low as 100 pg/ml. The activation of MO by LPS-LBP complexes was inferred to be mediated by CD14 based on experiments using anti-CD14 monoclonal antibodies. Biochemical evidence for complexation of LPS-LBP complexes with CD14 has now been obtained using ^{125}I-ASD-LPS, a photoactivatable, reductively cleavable derivative of LPS. From a mixture of CD14 expressing cells, ^{125}I-ASD-LPS and LBP, ^{125}I-CD14 may be immunoprecipitated after photolysis and CD14 is the major cell surface protein which becomes labelled.

Candidates for Lipid A Receptors

CB 007 IDENTITY OF PEPTIDOGLYCAN AND LIPOPOLYSACCHARIDE RECEPTORS, Roman Dziarski, Indiana University School of Medicine, Gary, IN 46408.

We have identified binding sites for peptidoglycan (PG), the major cell wall constituent of all bacteria and a macrophage and polyclonal B cell activator, on mouse lymphoid cells using rosetting and radioligand binding assays. This PG binding was specific, saturable, and had K_D = 0.12-0.46 μM. The specific binding was to the surface of the cells and did not represent endocytosis; was proportional to the number of cells and was completed within 20 min; was not changed by chaotropic agents, higher salt concentration, or chelating extracellular Ca^{2+}; and was not mediated by Ig or C. PG specifically bound to B cells, B cell cell lines, T cells, cloned T cells, and macrophage cell lines. Using [^{125}I]ASD-labeled affinity purified soluble and high M_r PG and photoaffinity crosslinking followed by 2D-PAGE and autoradiography, we have recently identified one dominant 70 kDa 6.5 pI PG-binding protein, present on mouse B and T lymphocytes and macrophages, but not on erythrocytes. The binding was specific for polymeric PG and was competitively inhibited by unlabeled PG (IC_{50} = 0.38 μM), and partially inhibited by dextran sulfate (IC_{50} = 124 μM), *O*-acetylated PG monomers (IC_{50} = 521 μM), and $(GlcNAc)_3$ (IC_{50} = 10 mM), and was not inhibited by non-*O*-acetylated PG monomers and dimers, muramyl dipeptide, PG pentapeptide, GlcNAc, teichoic acid, protein A, and other unrelated compounds. The cell surface location of the 70 kDa PG-binding protein was indicated by the ability of PG to bind to this protein in intact metabolically inactive cells (formalin-fixed cells or to viable cells at 4°C in the presence of 0.1% NaN_3), and by the ability to extract the 70 kDa PG-binding protein from viable B lymphocytes by non-cytotoxic concentration of n-octyl-ß-D-glucopyranoside.

Furthermore, we have shown that both PG and LPS bind to the same dominant 70 kDa 6.5 pI protein on the surface of mouse B lymphocytes, which is identical with the 73 kDa LPS receptor discovered by Lei and Morrison. This conclusion was supported by the following results: (a) the 70 kDa PG- and LPS-binding proteins comigrated following photoaffinity crosslinking and 2D-PAGE; (b) crosslinking of PG to this 70 kDa protein was competitively inhibited by LPS (IC_{50} = 7.3 μM), LPS from a deep rough mutant (IC_{50} = 6.9 μM), and lipid A (IC_{50} = 18-72 μM); (c) crosslinking of LPS to this 70 kDa protein was competitively inhibited by polymeric soluble PG (IC_{50} = 0.09 μM) and sonicated high M_r PG (IC_{50} = 0.6 μM); (d) crosslinking of both PG and LPS to this 70 kDa protein was also competitively inhibited by dextran sulfate (IC_{50} = 115-124 μM); (e) crosslinking of both PG and LPS to this 70 kDa protein was inhibited by a $(GlcNAc)_2$-specific lectin; (f) peptide maps of the 70 kDa proteins digested with chymotrypsin, subtilisin, staphylococcal protease V, or papain were identical for PG- and LPS-binding proteins and unique for each enzyme; and (g) anti-PG-LPS receptor mAbs bound to one 70 kDa protein and to cells that express the 70 kDa PG-LPS receptor, but not to cells that do not express this receptor, and binding of these mAbs to cells was inhibited by both LPS (IC_{50} = 20-80 nM) and soluble PG (IC_{50} = 10 nM). Binding of PG to the 70 kDa protein was 20 to 1200 times stronger than the binding of LPS or lipid A on per mol basis, but when aggregated micellar structures of LPS or lipid A were considered, the avidities of LPS and PG binding were similar.

These results indicate that both PG and LPS bind to the same 70 kDa receptor protein which is specific for the $(GlcNAc\text{-}MurNAc)_n$ backbone of PG and the $(GlcNAc)_2$ part of lipid A.

CB 008 LIPID A BINDING PROTEINS IN 70Z/3 CELLS. Theo N. Kirkland[1], Frances K. Multer[1], Peter S. Tobias[2], and Richard J. Ulevitch[2], [1]VA Medical Center and the University of California, San Diego, San Diego, CA 92161 and [2]Research Institute of Scripps Clinic, La Jolla, CA 92037.

The mouse pre-B cell line 70Z/3 responds to lipopolysaccharide (LPS) by transcriptional activation of the kappa light chain gene and expression of surface IgM. We have identified cellular proteins which specifically bind LPS using a ^{125}I-labelled photoaffinity derivative of Salmonella minnesota Re595 LPS (^{125}I-ASD-Re595), analyzing the labelled proteins by SDS-PAGE and autoradiography. ^{125}I-ASD-Re595 labelled several major proteins in 70Z/3 cells. The most prominently labelled protein had an apparent molecular weight of 18 kDa; a less prominently labelled band was also seen at 25 kDa. Neither one of these correlated with a major Coomasie-Blue stained protein. Labelling of both of these bands was inhibited by a 10-100 fold excess of un-derivatized LPS. Labelling of the 18 and 25 kDa bands was observed at concentrations of ^{125}I-ASD-Re595 as low as 0.15 μg/ml; half maximal labelling occurred at 0.6 μg/ml. Considering the low efficiency of the derivatization and photoaffinity labelling reactions (probable efficiency < 1%), this correlates well with the maximally stimulatory concentration of LPS for 70Z/3 cells. Maximal labelling of the 18 and 25 kDa bands occurred within 30 minutes. The labelled proteins appeared to be on the surface of the 70Z/3 cell because they co-purified with the plasma membrane and could be removed from intact cells by trypsinization. Biologically active LPS and lipid A molecules inhibited labelling as effectively as Re595 LPS; one biologically inactive lipid A molecule was a very poor inhibitor of labelling. These results suggest that the 18 and 25 kDa surface proteins may play a role in activation of 70Z/3 cells by LPS.

CB 009 GENETIC AND BIOCHEMICAL APPROACHES TO UNDERSTANDING MACROPHAGE LPS RECEPTOR, Masahiro Nishijima, Sayuri Hara-Kuge, Fumio Amano, and Yuzuru Akamatsu, Department of Chemistry, National Institute of Health, Tokyo, JAPAN

Bacterial lipopolysaccharide (LPS), along with proteins and phospholipids, is a major component on the outer surface of the outer membrane of Gram-negative bacteria. LPS triggers many pathophysiological events in mammalian cells, including the activation of macrophages or cultured macrophage-like cells such as J774.1 cells. LPS consists of a complex oligosaccharide covalently bound to lipid A, which is now believed to be the endotoxically active center of different LPS preparations. Although the physiological effects of LPS or lipid A on macrophages have been extensively studied, the actual biochemical and molecular mechanisms underlying the activation of macrophages by LPS remain obscure. Despite many extensive works, the existence of LPS-receptor(s) which triggers the initial response of macrophages to LPS, for example, has not yet been proved.

One strategy to identify LPS-receptor(s) on macrophages is the use of somatic cell mutants defective in LPS-binding. For this purpose, we isolated LPS-resistant mutants which did not respond to LPS from a macrophage-like mouse cell line, J774.1. Unlike the parental J774.1 cells, these mutants grew even in LPS-added medium as well as normal growth medium without any morphological changes. Assay of ^{125}I-LPS binding to the cell monolayers revealed that one of these LPS-resistant mutants (LR-9) was strikingly defective in LPS-binding activity. Scatchard plot showed that LR-9 cells lacked the high affinity binding sites which were present in J774.1.

LPS enhanced O2-generation and the release of arachidonic acid in J774.1 cells but not in LR-9 cells. Other stimulants such as zymosan and TPA, however, induced the release of arachidonic acid in LR-9 cells as well as in J774.1 cells. These results suggest that LR-9 cells specifically lack the ability to respond to LPS.

To identify the LPS receptor(s), photoactivatable and cleavable ^{125}I-ASD-LPS was cross-linked to proteins on the surface of J774.1 cells. Two distinctive radioactive bands, corresponding to molecular masses of approximately 55- and 65-kDa, were observed on autoradiography, which did not appear when binding was competed by unlabeled LPS. Unlike in the case of J774.1 cells, both the 55- and 65-kDa bands were not detected when LR-9 cells were treated with ^{125}I-ASD-LPS as decribed above. It is, therefore, very likely that one or both of the two proteins might relate to the specific membrane receptor for LPS. The 55-kDa protein could be CD-14 which is a 55-kDa glycoprotein and has been shown to be a receptor for complexes of LPS and LPS binding protein (LBP) present in serum.

CD14 and A New Paradigm for LPS Recognition By Cells

CB 010 GLYCOSYL PHOSPHATIDYLINOSITOL (GPI) LINKED RECEPTORS ARE SEQUESTERED IN *CAVEOLAE*, THE THIRD COATED PIT, K.G. Rothberg[1], W.-J. Chang[1], Y.-Y. Ying[1], J.R. Glenney[2], B.A. Kamen[1], and R.G.W. Anderson[1]. Department of Cell Biology and Neuroscience, University of Texas Southwestern Medical Center, Dallas, TX 75235[1], and Department of Biochemistry, University of Kentucky College of Medicine, Lexington, KY 40536[2].

Internalization of 5-methyltetrahydrofolate utilizes a novel pathway that does not merge with the clathrin coated pit pathway. The GPI-linked folate receptor is sequestered on the plasma membrane in numerous discrete clusters that are associated with caveolae. Caveolae have been implicated as the molecular vehicles for transcytosis of macromolecules across endothelial cell but are found in all cells. The maintenance of the structural integrity of the caveolae as well as the organization of the GPI linked receptors is dependent on the presence of cholesterol in the membrane. Furthermore, the rate of internalization of folate and recycling of the GPI linked receptors is altered in cholesterol depleted membranes. Using rapid freeze, deep etch microscopy, we have shown that the caveolae are decorated on their cytoplasmic surface by a special set of filaments that form a striated coat. The filamentous coat cannot be removed by high salt wash, but exposure to cholesterol binding drugs causes invaginated caveolae to flatten out and the filaments to disassemble. Antibodies directed against p22, a 22 KDa substrate for *v-src* tyrosine kinase in virus transformed chick embryo fibroblasts, decorate the filaments, which suggests that this molecule is a structural component of the coat. We have named p22, *caveolin*. Preliminary data suggest that caveolin is post translationally modified by covalent addition of fatty acids. The caveolae are the third member of coated pit specializations that function to initiate molecular transport.

CB 011 EXPRESSION AND FUNCTION OF HUMAN CD14 IN TRANSGENIC MICE, Sanna M. Goyert[1], Dan Jiao[1], Ben-Z. Tsuberi[1], Pilar Garin-Chesa[2], Luisa Tesio[1], Guo Wei Rong[1], Alain Haziot[1] and Enza Ferrero[1], [1]North Shore University Hospital/Cornell University Medical College, Manhasset, NY 11030, [2]Memorial Sloane-Kettering Cancer Center, New York.

In vitro studies have recently shown that CD14 is a receptor for LPS when LPS is complexed to the acute phase protein LBP (LPS binding protein). In order to study the effects of CD14 *in vivo* we have produced transgenic mice which have integrated the human CD14 gene. FACS analyses and immunochemical staining of tissue sections show that these mice express high levels of human CD14 on their monocytes and granulocytes. As a result of this expression, these mice are dramatically more susceptible than normal mice to LPS-induced endotoxin shock. These mice provide a new biological model for the study of the response to LPS through the human CD14 gene and furnish direct, *in vivo* evidence of the influence of expression of CD14 on the induction of endotoxin shock. In addition, these mice provide a unique model for the testing of therapies directed against the human CD14 protein.

CB 012 GPI-ANCHORED PROTEINS AS TRANSDUCING RECEPTORS, Peter J. Robinson, Margaret Millrain and Rosemarie Hederer, Transplantation Biology, Clinical Research Centre, Watford Road, Harrow, Middlesex. HA1 3UJ. U.K.

Glycosylphosphatidylinositol (GPI) is frequently used as an alternative anchor for eukaryotic cell surface proteins. The GPI-anchor core is remarkably conserved in all species studied so far, including yeast and man, suggesting strong evolutionary pressure to maintain this configuration. GPI-anchors have a number of properties of potential biological importance which are not shared with transmembrane protein anchors, such as a higher translational mobility and susceptibility to cleavage by phospholipases C and D. In addition, many GPI-anchored proteins are potent signal transducing molecules. Activation of hematopoietic cells by antibodies against GPI-anchored proteins results in growth, cell division and lymphokine production, suggesting that signalling is an important biological property of these anchors.

Using a transgenic mouse system, we have shown that activation of resting T lymphocytes by antibodies specific for Qa-2, a GPI-linked class I transplantation antigen, can be abrogated if the GPI-anchor is replaced by a transmembrane protein domain. This indicates that the anchor is directly involved in signal transduction.

In resting T-lymphocytes, other signals in addition to antibody are usually required to achieve proliferative responses. Thus, Qa-2 specific antibodies require cross-linking with anti-immunoglobulin and addition of phorbol esters. Certain Thy-1 specific antibodies, however, are able to act alone to induce T cell proliferation. We have observed that these antibodies induce the formation of extensive tight intercellular junctions with a regular spacing of 12-14 nm. Other activating antibodies do not form homogeneous junctions. We have evidence that the degree of junction formation is correlated with activating capacity and is clearly epitope dependent.

A number of groups have demonstrated association of Thy-1 with other proteins, both on the external face of the cell membrane and on the cytoplasmic side. These results suggest the existence of a transmembrane signalling complex which is able to translate surface stimuli into biochemical responses. This complex appears to perform three distinct functions: (i) association of the anchor with the complex via an anchor-binding protein, (ii) linkage of this protein to the cytoskeleton and (iii) association of the complex with a transducer e.g. a tyrosine kinase. We are attempting to systematically dissect this complex using a combination of affinity chromatography and microsequencing techniques. We aim to reconstitute the complex from isolated components as has been done with the T lymphocyte antigen receptor.

LPS-Induced Cell Stimulation Via CD14

CB 013 ANALYSIS OF LPS-PMN INTERACTION IN BLOOD, Larry A. Sklar, Scott Simon, Mary-Margaret Brandt, and Eric Lynam, Cytometry, University of New Mexico School of Medicine, Albuquerque, NM 87131 and Ronald Weingarten, John C. Mathison, Richard J. Ulevitch and Peter S. Tobias, Immunology, Scripps Research Institution La Jolla, CA 92037.

An analysis of the expression of receptors on the neutrophil cell surface yields insight into the activation and function of neutrophils in host defense. Agents that regulate the expression of neutrophil surface receptors have profound implications in the management of the host response to infection. Because, neutrophils are exquisitely sensitive to the conditions of their isolation, we have analyzed the regulation of neutrophil receptor expression using flow cytometry in whole blood, allowing the discrimination of neutrophils from other blood constituents without isolation. We utilized a panel of anti-CD14 mAbs to document the presence of CD14 on the cell surface of polymorphonuclear leukocytes. In whole blood neutrophils express low, but detectable levels of CD14 and CR3 and substantially increase cell surface CD14 and CR3 in response to formyl peptide and LPS. The increases occurred in parallel, were independent of protein synthesis and could be blocked by mAb 3C10 against CD14. These findings suggest that CD14, like CR3, is mobilized from an intracellular compartment to the neutrophil plasma membrane in response to inflammatory stimuli. Since mobilization is in direct response to the action of LPS on neutrophil CD14, CD14 appears to play a role in the host response to gram-negative sepsis and endotoxemia. We have extended previous observations of the ability of methylprednisolone sodium succinate (MPSS) to inhibit mobilization of intracellular receptors. MPSS blocks receptor upregulation in response to three different stimuli (formyl peptide, LPS, and GM-CSF). MPSS reversibly inhibits upregulation of CR3, formyl peptide receptor, and CD14 in response to each of the three stimuli in a dose dependent manner. Because these receptors are likely to function at least in part through independent signalling pathways, we speculate that MPSS is functioning at a common site. Further investigation is needed to determine the specific means by which corticosteroids interfere with neutrophil upregulation mechanisms.

CB 014 A NEW MODEL FOR RECEPTOR MEDIATED STIMULATION OF MACROPHAGES (MO) BY BACTERIAL LIPOPOLYSACCHARIDE (LPS). R.J. Ulevitch, J.C. Mathison, J.-D. Lee, K. Kato, D. Mintz, and P.S. Tobias. The Scripps Research Institute, La Jolla, CA 92037.

We have defined a receptor mediated pathway of LPS-induced MO stimulation that involves LPS binding protein (LBP), the MO membrane protein CD14 and a high-affinity membrane receptor for lipid A. This model predicts that MO contain a pool of lipid A receptors that can be modulated by engagement of CD14 by LPS-LBP complexes, providing an explanation for a variety of MO responses including tolerance and cytokine production. LBP is a plasma protein that forms high affinity complexes with LPS, binds to MO CD14 and causes marked enhancement of cytokine production. This is due to increased MO sensitivity to LPS and more rapid production and enhanced stability of cytokine mRNA. In contrast LBP has no effect on cytokine production induced by PMA or heat-killed S.aureus. We will present evidence for phosphoprotein intermediates in signal transduction via the LBP/CD14-dependent pathway. We have defined interactions of LPS-LBP complexes with CD14 and the putative lipid A receptor using photochemical cross-linking reagents. Transfection of CD14 into LPS-responsive, CD14-negative cell lines markedly increases their responsiveness to LPS, providing additional evidence for this model. Thus we hypothesize that the LPS receptor on MO consists of at least two components, CD14 and an as yet unidentified lipid A binding protein.

CB 015 NOVEL PLASMA PROTEINS THAT MEDIATE INTERACTION OF LPS WITH CD14. Samuel D. Wright, David Miller, Leslie A. Leonard, Thierry Calandra,and Mark Wurfel, Laboratory of Cellular Physiology and Immunology, Rockefeller University, 1230 York Ave., New York, NY 10021.

Our previous studies have shown that LPS binding protein (LBP) binds to LPS on the surface of erythrocytes (ELPS) and that the resulting opsonized erythrocytes bind to human macrophages (MO) through an interaction with CD14. Using assays of this opsonic activity we found that serum or plasma from healthy donors had half-maximal activity at dilutions of 1:4000. This is 2-3 logs higher than expected from the LBP content of these sera, suggesting the presence of proteins in addition to LBP that mediate interaction of LPS with CD14. The opsonic activity of plasma was partially purified with high yield on BioRex 70 resin, then further fractionation on Mono Q. We were surprised to find that none of the individual peaks from a Mono Q column were active in our assay, but activity could be nearly completely recovered by combining several fractions together. Thus at least two distinct species must be simultaneously present to mediate interaction of LPS with CD14. Further studies suggest that these distinct species interact as protease and substrate. Opsonic activity of plasma was completely blocked with reagents that inhibit serine esterases while the opsonic activity of LBP was unaffected by these reagents. Thus, the major factor in plasma capable of mediating interaction of ELPS with MO has characteristics and a mode of action distinct from LBP. We have named his activity, "septin."

Septin clearly interacts with CD14 since opsonized ELPS bind to CD14-bearing transfectants and all interaction with MO can be blocked with anti-CD14. Though Septin entails one or more proteolytic activities, these are distinct from the proteolytic activities of either the clotting or complement cascades.

Septin causes not only binding of LPS to CD14 but also stimulation of cells. PMN were not stimulated by spreading on LPS-coated plastic wells or serum-coated wells, but pre-incubation of serum with an LPS-coated surface yielded a substrate that dramatically activated the binding of activity of CR3 on PMN. All of the stimulatory activity of serum was inhibited by blockers of proteolysis. In a similar fashion, monocytes secreted TNF after spreading on LPS-septin surfaces, but failed to secrete TNF upon spreading on LPS-coated or serum-coated surfaces and failed to secrete TNF if protease inhibitors were added to the plasma during incubation with the LPS substrate.

These results suggest that plasma contains one or more proteolytic activities that function, perhaps in a cascade, to mediate the interaction of LPS with CD14. Preliminary studies on the biochemistry of these species will be discussed. Supported by USPHS AI22003 and AI30556.

Signal Transduction Mechanisms of LPS

CB 016MARCKS, A MYRISTOYLATED PROTEIN KINASE C SUBSTRATE, TRANSDUCES LPS-DEPENDENT RESPONSES IN MACROPHAGES AND NEUTROPHILS. Alan Aderem*, Marcus Thelen*, Antony Rosen*, John Seykora*, Angus C. Nairn*, Paul A. Janmey#, and John H. Hartwig#. *The Rockefeller University, 1230 York Avenue, New York, NY 10021 and #Department of Anatomy and Cellular Biology, Harvard Medical School, Boston, MA 02115.

LPS has the capacity to prime macrophages and neutrophils for potentiated protein kinase C (PKC)-dependent signals such as those leading to arachidonic acid metabolism and cell motility. It does so, in part, by inducing the synthesis of PKC substrates which are otherwise limiting within the cell. Our focus has been the molecular characterization of a family of myristoylated PKC substrates whose transcription, translation and phosphorylation is regulated by LPS and which therefore represent ideal candidates as effector molecules of LPS-induced responses. These LPS-induced substrates include a 68K protein known by the acronym MARCKS (for myristoylated, alanine-rich C kinase substrate). MARCKS is a major, specific substrate of PKC which is phosphorylated during macrophage and neutrophil activation, growth factor-dependent mitogenesis and neurosecretion. MARCKS is also a calmodulin-binding protein and binding of calmodulin inhibits phosphorylation of the protein by PKC. Several recent observations from our laboratory suggest a role for MARCKS in cellular morphology and motility. First, in macrophages MARCKS locates at points of cellular adherence where actin filaments insert at the plasma membrane and is released to the cytoplasm upon activation of PKC. Second, during neutrophil chemotaxis MARCKS undergoes cycles of phosphorylation-dependent release from, and reassociation with, the plasma membrane. Third, *in vitro* MARCKS is an F-actin crosslinking protein whose activity is inhibited by PKC-mediated phosphorylation and by binding to calmodulin. MARCKS, therefore, appears to be a regulated crossbridge between actin and the plasma membrane. Regulation of the plasma membrane- and actin- binding properties of MARCKS represents a convergence of the PKC and calmodulin signal transduction pathways in the control of actin cytoskeleton/plasma membrane interactions.

CB 017 INTERACTIONS OF BACTERIAL LIPOPOLYSACCHARIDE WITH MICROTUBULE PROTEINS. Aihao Ding and Carl Nathan. Cornell University Medical College, New York, NY 10021.

The mechanisms by which bacterial lipopolysaccharide (LPS) stimulates immune cells are unknown. A possible role for microtubule proteins in LPS actions has been indicated by our previous findings that the microtubule-active agent, taxol, can mimic effects of LPS in macrophages from normal strains of mice, but not from genetically LPS-hyporesponsive strains (Science, 248:370). Interactions of microtubule proteins and LPS can be demonstrated in vitro using microtubules isolated from mouse brain by: (1) co-elution of LPS and tubulin dimer through a gel filtration column, (2) blotting of microtubule proteins with labeled LPS in nitrocellulose membranes, and (3) crosslinking of microtubule proteins and LPS with an iodinatable and photoreactive bifunctional crosslinker - sulfosuccinimidyl 2-(*p*-azidosalicylamido) ethyl-1,3'-dithiopropionate. The results from crosslinking experiments suggested that β-tubulin and microtubule associated protein-2 (MAP-2), a predominant MAP in the brain, bound LPS specifically. Crosslinking was inhibited by an excess of unlabeled LPS or lipid A, but not by 2 M NaCl. Under the same conditions, neither myosin nor soybean trypsin inhibitor was labeled by the LPS probe. LPS appears to bind polymerized tubulin preferentially since millimolar Ca^{2+} but not Mg^{2+} inhibited crosslinking of LPS and tubulin, correlating with the effect of these divalent cations on tubulin assembly. These findings support the hypothesis that the microtubule network could be an intracellular target for LPS, and imply that a β-tubulin-associated MAP in macrophages could have an important role in LPS actions.

CB 018 REGULATION OF LPS-INDUCED GENE EXPRESSION, Nigel Mackman, The Scripps Research Institute, La Jolla, CA 92037.

Lipopolysaccharide (LPS) activation of cells of monocytic lineage leads to rapid and transient expression of a set of inflammatory gene products, including tissue factor (TF). This transmembrane receptor is the major cellular initiator of the blood coagulation cascades. Pathologic activation of the coagulation cascades by TF within the vasculature has been implicated in several disease states, including septic shock and various thromboembolic diseases. The TF gene is transcriptionally silent in monocytes but responds rapidly to the agonist LPS. We have been analyzing transcriptional regulation of the TF gene in the THP-1 cell line, as a model of monocytic differentiation, and induction in response to LPS. After stimulation, the rate of transcription of the TF gene is increased and there is a transient accumulation of the mature 2.2-kb TF mRNA, which is maximal at 2 h. In addition, we observed a significant modulation of TF mRNA stability: at 1 h after LPS stimulation, TF mRNA is stable during a 60-min period and has a half-life of >120 min, whereas at 2 h, the half-life declines to 25 min. These results demonstrate that LPS-induced accumulation of TF mRNA levels in these monocytic cells is accomplished by both transcriptional and post-transcriptional control mechanisms. Functional studies of the 5' promoter region led to the identification of a 56-bp LPS response element (LRE) that confers LPS responsiveness to a heterologous promoter. LPS stimulation of these cells activated at least two distinct proteins that were found to bind to nucleotide sequences within the LRE that resemble consensus binding sites for the nuclear transcription factors AP-1 and NF-κB. Induction of the TF gene may represent a prototypic example of gene activation in monocytic cells by assembly of transcription factor complexes, and may clarify the role of AP-1 and NF-κB in the regulation of other LPS-responsive genes.

CB 019 LPS INDUCED PRIMING OF PHAGOCYTIC CELLS: INACTIVATION OF LPS BY NEUTROPHILS,
Michael J. Pabst, Karen M. Pabst, Demin Wang, and Yoshitomi Aida,
Dental Research Center and Department of Biochemistry, The University of Tennessee, Memphis.

LPS primes neutrophils for enhanced release of microbicidal oxygen radicals like superoxide. Factors from serum appear necessary for low concentrations of LPS to prime neutrophils. We have found that if neutrophils were carefully washed to remove all traces of serum, then the neutrophils responded weakly or not-at-all to LPS in the concentration range of 2 ng/ml and below. Our experiments suggested that serum factors perform two functions. The first function of serum factors is to bind LPS to form a complex that is recognized by neutrophil receptors. For example, LPS binds LPS Binding Protein to form a complex that is recognized by the neutrophil membrane glycoprotein CD14. We believe that a second function of serum proteins is to protect LPS against inactivation by neutrophil enzymes. Medium containing 1 ng/ml of LPS or lipid A from *E. coli* was incubated with 5 million neutrophils per ml for 10 minutes in the absence of serum, then the LPS-containing medium was removed and tested on fresh neutrophils in the presence of 0.5% heat-inactivated serum. The LPS or lipid A that had been exposed to neutrophils showed greatly reduced activity in priming the fresh neutrophils. In contrast, LPS or lipid A that was pre-incubated with neutrophils in the presence of 0.5% serum was fully active when tested on fresh neutrophils. Potential explanations for this effect are that LPS was absorbed by the first set of neutrophils, or alternatively that the LPS was chemically modified by the first set of neutrophils. Preliminary experiments with radioactive LPS indicated that almost all of the LPS remained in the medium, suggesting that LPS was not absorbed by neutrophils. There are precedents for two chemical modifications of LPS that would render the LPS inactive in priming neutrophils. The first modification is removal, by the neutrophil enzyme, acyloxyacyl hydrolase, of fatty acids linked to the beta hydroxy fatty acids in the lipid A moiety of LPS. However, preliminary experiments with LPS specifically labelled in the beta linked fatty acids did not indicate significant deacylation of LPS. The second potential chemical modification is loss of a phosphate group from the lipid A moiety of LPS to yield an LPS containing monophosphoryl lipid A. Native diphosphoryl lipid A primes neutrophils, whereas monophosphoryl lipid A does not. Our hypothesis is that a phosphatase on the neutrophil surface is capable of removing phosphate from LPS to form an LPS that no longer primes neutrophils. We further propose that serum factors prevent this dephosphorylation. We are currently attempting to identify the chemical change that occurs in lipid A that has been exposed to neutrophils in the absence of serum. (Supported by NIH grant, DE05494.)

Effector Mechanisms for LPS Action

CB 020 LPS INDUCTION OF THE COAGULATION PROTEASE CASCADES: THE TISSUE FACTOR (TF) PATHWAYS, Thomas S. Edgington, The Scripps Research Institute, La Jolla, CA 92037.

LPS induces expression of TF on the surface of monocytes and endothelial cells. TF is the high affinity receptor and requisite cofactor for the latent serine protease factor VIIa. The binary complex TF·VIIa is catalytically active and possesses extended substrate recognition for factors X and IX. As a result both of these zymogens are proteolytically activated to the serine proteases Xa and IXa, driving the extrinsic prothrombinase complex amplification and intrinsic (factor VIIIa) pathway, both culminating in thrombin generation. The pathways are regulated by inhibitors as well as positive feedback activation of VII to VIIa and XI to XIa. The structural basis of TF function derives from the paired non-symmetrical binding of VII and VIIa to TF to form the functional TF·VIIa complex, a bimolecular assembly which has been mapped by chemical cross-linking and microsequencing. The effects of inhibition of TF function on in vivo challenge with LPS and of fatal septic shock are to diminish the in vivo activation of the coagulation pathways, the consumption coagulopathy, and the lethality of the baboon model. TF is distantly homologous to other cytokine receptors and growth hormone related receptors as a superfamily. Rules for function of the Cytokine Receptor Family are suggested from the assembly rules for TF.

CB 021 EXPRESSION OF INTERLEUKIN-8 (IL-8) AND MONOCYTE CHEMOATTRACTANT PROTEIN-1 (MCP) VIA LPS-DEPENDENT CYTOKINE NETWORKS. Steven L. Kunkel and Robert M. Strieter, Departments of Pathology and Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan 48109-0602

The evolution of an inflammatory reaction involves a dynamic series of cellular events controlled by specific signals which are important to the initiation, maintenance, and final resolution of the response. This progression is influenced by the expression and subsequent regulation of key mediators which play a predominant role during a particular stage of the developing inflammatory lesion. For example, the recruitment of leukocytes from the vascular compartment to an area of inflammation is a complex event that appears to be dependent upon early response mediators which are expressed during the initiation phase of inflammation. These proximal cytokines, include both interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF), play a key role during the initiation of immune reactivity by promoting endothelial cell/leukocyte interactions. The physical contact and initial binding of leukocytes to the endothelium is known to be mediated, in part, by IL-1/TNF induced adherence proteins. Although the mechansims responsible for adhering leukocytes to the vascular wall is becoming increasing clear, the remaining events needed to move the cells from the lumen of the vessel to an inflammed area are not entirely known. Past studies have associated in vivo chemotactic activity with a number of biologically active compounds; yet these same factors are not true chemotaxins when assessed in vitro. For example, the intradermal administration of either IL-1, TNF, or lipopolysaccharide (LPS) can elicit the recruitment of neutrophils within hours to the injection site, but these inflammatory mediators fail to induce chemotactic activity in vitro. Therefore, the ability of either IL-1, TNF, or LPS to initiate a chemotactic response in vivo is dependent upon the synthesis of de novo chemotactic factors. The recent discovery of a novel group of chemotactic cytokines, including interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP) has partially clarified some of the confusing obsevations in this area. These chemotactic cytokines are likely instrumental in localizing inflammation to a particular tissue site, as these factors are the product of many cells, including fibroblasts, hepatocytes, and epithelial cells. Interestingly, these cells do not respond directly to LPS, but are dependent upon host generated IL-1 or TNF. Thus, one scenario for LPS-dependent elicitation of leukocytes to an area of inflammation is via cytokine networks, whereby LPS can serve as a stimulus for resident macrophage-derived IL-1 or TNF, which can in-turn cause the expression of IL-8 or MCP by resident non-immune cells. We have studied this hypothesis by treating normal resident human alveolar macrophages with LPS, recovering the conditioned media, and using this conditioned media to stimulate IL-8 mRNA expression by human fibroblast or epithelial cells. LPS-conditoned media resulted in an 80% increase in the expression of IL-8 mRNA, as compared to non-LPS conditioned media. To ascertain the contribution of LPS-induced, macrophage-derived IL-1 or TNF to IL-8 expression by fibroblasts or epithelial cells, antibody neutralization studies were performed. LPS-stimulated macrophage conditioned media treated with antibody against human IL-1 or TNF resulted in a 45% and 28% reduction in the expression of fibroblast-derived IL-8. In contrast, control antibody did not alter IL-8 generation from these non-immune cells. A common theme that is evolving from thses studies is that resident non-immune cells can no longer be viewed as passive 'bystanders' during inflammation, but must be viewed as active effector cells that can contribute to the initiation and maintenance of leukocyte recruitment.

New Opportunities for Therapy-I

CB 022 DEVELOPMENT AND CHARACTERIZATION OF MURINE MONOCLONAL ANTIBODIES DIRECTED AGAINST *ENDOTOXIN*, David L. Dunn, Department of Surgery, Division of Surgical Infectious Diseases, University of Minnesota, Minneapolis, MN 55455

Increasing evidence has indicated that gram-negative bacteremia is associated with concurrent endotoxemia, and that even low level endotoxemia can provoke a markedly exaggerated host response in which macrophage activation clearly plays an early pivotal role. Gram-negative lipopolysaccharide (LPS, endotoxin) is perhaps the most potent stimulus of macrophage monokine production, that consists of the secretion of tumor necrosis factor, interleukin-1, and interleukin-6. For that reason, the ability of anti-LPS antibody to exert protective capacity during gram-negative bacterial sepsis is being intensively examined. Whereas the lipid A (LA) portion of LPS has been associated in experimental models with the majority of toxic effects, the host humoral response to gram-negative bacteremia or endotoxemia is largely serotype specific and is not directed against the LA moiety. Although the core region of LPS consists of three regions [outer, intermediate (inter), and inner or deep), only the deep core/lipid A (DCLA) region of LPS appears to represent a highly biochemically and immunologically conserved region of the LPS molecule, and is thus an ideal candidate against which cross-reactive antibody with anti-toxin activity could be developed. The DCLA antigenic region is expressed extensively on the cell surface of rough mutants of Escherichia coli and Salmonella minnesota. These organisms or their derived outer membrane LPS thus represent suitable immunogens for cross-reactive antibody production. For that reason, the ability of anti-DCLA antibody to act in conjunction with standard therapeutic modalities has been examined. Several problems, however, exist at present: 1) the exact binding site within the DCLA region that will serve to maximize cross-reactivity and protection has not been established, 2) the extent to which anti-DCLA monoclonal antibodies (mAbs) bind to intact bacteria or LPS in vitro and in vivo has not been defined, 3) in vitro assays that will serve as predictors of in vivo protective capacity have not been developed, and 4) the mechanism by which anti-LPS mAbs provide protection in vivo has not been defined. In order to examine this problem, anti-LPS mAbs were developed from fusions of splenocytes (derived from Balb/C mice immunized with either killed gram-negative bacteria or their derived LPS) and the P3X63-Ag8.653 murine myeloma cell line. Initial hybridoma screening took place against the primary immunogen using ELISA and immunodot blot techniques, and Western immunotransblot was used to precisely define binding specificity.

O	CORE: Outer	Inter	Deep	LA	BINDING SITE	SMOOTH LPS BINDING
+	-	-	-	-	O ANTIGEN	YES
+	+	-	-	-	OUTER CORE	YES
-	+	-	-	-	OUTER CORE	NO
-	-	+	+	-	INTERMEDIATE CORE	NO
-	-	+	-	-	INTERMEDIATE CORE	NO
-	-	-	+	-	INNER CORE	NO
-	-	-	-	+	LA	NO
-	-	-	+	+	INNER CORE/LA	NO
+	+	+	+	+	INNER CORE/LA	YES

Although the basic concept that the DCLA region of LPS represents an extensively shared antigenic site appears to be valid, the unexpected occurrence of apparently unique antigenic sites within the intermediate core, the deep core, and the lipid A regions of LPS has been substantiated. Apparently there exist both "public" (i.e. shared) and "private" (i.e. unique) specificities within all these regions of the LPS molecule. Appropriate selection of highly cross-reactive mAbs must therefore be accomplished by *initial* screening of hybridomas against various core and LA epitopes.

CB 023 PRECLINICAL AND CLINICAL PROPERTIES OF THE ANTI-LPS MONOCLONAL ANTIBODY E5®, Nancy Wedel[1], Brian Parent[1], Paul Conlon[1], Helene Gazzano-Santoro[1], Steven Opal[2], Patrick Trown[1], [1]XOMA Corporation, Berkeley, CA 94710, [2]Brown University and Memorial Hospital, Providence, RI 02912.

The abundant evidence that endotoxin is a key mediator in the adverse sequelae of Gram-negative sepsis has led to development of antiendotoxin monoclonal antibodies (MAb) in an attempt to improve therapy for this frequently lethal condition. Antiendotoxin antibody E5®, a murine monoclonal IgM directed against lipid A, has been tested in a variety of *in vitro* systems and animal models, with results consistent with its efficacy in improving outcome from Gram-negative sepsis in a prospectively identifiable patient population.

By use of RIA, E5® has been shown to bind specifically and with high affinity (apparent KDs between 5-12 nM) to natural and synthetic Lipid A antigens, and to both rough and smooth LPS. By a capture/LAL assay, E5® has demonstrated pan-reactivity with numerous rough and smooth LPS, many derived from clinical isolates. The importance of this *in vitro* activity is reflected in the efficacy of E5® in a neutropenic rat model of *Pseudomonas aeruginosa* sepsis. When treated with E5® or irrelevant MAb at time of febrile response, survival was significantly enhanced in the group treated with E5®. In addition, 24 hours after antibody treatment, mean serum levels of circulating endotoxin and TNF-alpha were dramatically lower in E5®-treated animals.

Two large phase III clinical trials have demonstrated that E5® significantly improves complete resolution of organ failures commonly associated with sepsis in patients not in refractory shock who have organ failures present upon entry to these studies. Such patients are identifiable without waiting for culture results. When these patients had a microbiologically documented Gram-negative etiology for their sepsis (about two thirds of those with organ failures), E5® significantly enhanced survival.

The clinical efficacy of E5® is consistent with its *in vitro* binding and *in vivo* therapeutic characteristics.

CB 024 ANTI-LPS MONOCLONAL ANTIBODIES, Elizabeth J. Ziegler, Department of Medicine, University of California San Diego, UCSD Medical Center, San Diego, CA 92103.

Gram-negative sepsis is an increasing problem among hospitalized patients, and the death rate remains high despite modern methods of treatment. In patients with gram-negative bacteremia, endotoxin (LPS) in the circulation is an important trigger for the cascade of events leading to organ failure and death. Therefore, efforts were made to develop anti-LPS antibodies to try to improve the outcome in gram-negative bacteremia (GNB). Human polyclonal antibody against common LPS core determinants protects experimental animals against lethality from endotoxin and gram-negative infection and was shown in a randomized trial to prevent death in patients with GNB (1). Because vaccinating donors and giving human serum to patients is not practical, a human monoclonal antibody (mAb) against LPS was sought, which would be protective and could be easily standardized and produced in quantity. A human IgM with such properties was developed (2). This mAb, called HA-1A, is directed to an epitope on lipid A, the toxic part of LPS. HA-1A was studied in a randomized, double-blind, placebo-controlled trial in septic patients (3). Of 543 patients treated, 200 had GNB. In patients with GNB, HA-1A reduced mortality by 39% (from 49% placebo to 30% - HA-1A, $p = 0.014$). Protection from HA-1A was evident in patients with shock ($p = 0.017$). The efficacy of HA-1A was reflected in more rapid resolution of major septic complications ($p = 0.024$ at 7 days), and HA-1A recipients were more frequently discharged from the hospital alive than placebo recipients ($p = 0.038$). HA-1A was particularly effective in patients with endotoxemia at baseline and was associated with decrease in serum TNF levels at 24 hrs ($p < 0.05$). *In vitro* studies suggest that protection in man may be mediated in part by enhanced LPS clearance via complement receptors and by interference with LPS-induced cytokine activation. Human mAb HA-1A appears to be safe and effective for immunotherapy of gram-negative bacteremia and shock.

References

1. Ziegler EJ, McCutchan JA, Fierer J, *et al.* Treatment of gram-negative bacteremia and shock with human antiserum to a mutant *Escherichia coli*. N Engl J Med 1982; 307:1225-30.
2. Teng NNH, Kaplan HS, Hebert JM, *et al.* Protection against gram-negative bacteremia and endotoxemia with human monoclonal IgM antibodies. Proc Natl Acad Sci USA 1985; 82:1790-4.
3. Ziegler EJ, Fisher CJ, Sprung CL, *et al.* Treatment of gram-negative bacteremia and septic shock with HA-1A human monoclonal antibody against endotoxin. N Engl J Med 1991; 324:429-36.

New Opportunities for Therapy-II

CB 025 THE EFFECTS OF THE INTERLEUKIN-1 RECEPTOR ANTAGONIST IN ANIMAL AND HUMAN SEPSIS, Michael A. Catalano, M.D., Synergen, Inc., 1885 33rd Street, Boulder, CO 80301 USA

Interleukin-1 (IL-1) has been proposed to be one of the key mediators of the sepsis syndrome caused by endotoxin from gram-negative organisms. A naturally occurring human recombinant IL-1 receptor antagonist (hrIL-1ra) has been produced which specifically blocks the actions of both IL-1α and IL-1β but exerts no agonist activity. The effects of hrIL-1ra have been studied in animal models of sepsis and in the human sepsis syndrome. Safety, efficacy, kinetic and mechanistic data from these studies will be reviewed.

Late Abstract

CELL BOUND AND SOLUBLE TUMOR NECROSIS FACTOR RECEPTORS. David Wallach, Hartmut Engelmann, Dan Aderka*, Cord Brakebusch, Yaron Nophar and Oliver Kemper. *The department of Molecular Genetics and Virology, The Weizmann Institute of Science, Rehovot and *The Department of Medicine, Ichilov Hospital, Tel Aviv Medical Center, Tel Aviv, Israel.*

In view of a growing evidence for a key role of tumor necrosis factor (TNF) in the pathogenesis of many diseases, there are increasing attempts to find ways of inhibiting the function of this cytokine. Soluble forms of the receptors for TNF, found to be present in the serum and urine, compete for TNF with the cell-surface TNF receptors and can thus block its function[1-4]. These proteins are now being tested as inhibitors of TNF in several animal models of TNF pathogenicity. In a number of such models the soluble TNF receptors (sTNF-Rs) have already been found to provide some protection. Clues to more effective ways for applying the sTNF-Rs for therapy may perhaps be gained from the study of the physiological function of these proteins. So far there is just fragmentary information on that subject, relating to the following points:

Occurence of the sTNF-Rs: The sTNF-Rs are present constitutively in the serum, although at very low concentrations; their serum concentrations increase significantly in diseases of both an inflammatory and non-inflammatory nature and were reported to increase also after administration of TNF.

Mechanisms of formation: The sTNF-Rs are derived from the cell-surface receptors by a proteolytic cleavage mechanism which is subject to effective enhancement by certain physiological regulators. This cleavage mechanism and the structural elements in the TNF receptor taking part in it are distinct from those involved in TNF uptake and in the signalling by these receptors.

Effects: Beside their inhibitory effect on the function of TNF, which reflects their ability to bind TNF, the sTNF-Rs have also the ability to attenuate a spontaneous decay of TNF activity by stabilizing TNF structure within the complexes which they form with it. Thus, depending on the rate at which TNF is cleared from the site of its formation, the sTNF-Rs may, at different situations, affect the function of TNF in quite a different manner; in some situations they may inhibit the effects of TNF, in others, serve as carriers for TNF, and in some cases they may even augment the effects of TNF by prolonging its function.

1)Olsson, I. et. al (1989) Eur. J. Haematol. **42**:270.
2)Seckinger, P et al.(1989) J. Biol. Chem. **264**:11966
3)Engelmann, H. et al.(1989.)J. Biol. Chem. **264**:11974.
4)Nophar, Y.(.1990). EMBO J. **9**:3269.

LPS and LPS Binding Proteins

CB 100 *Abstract Withdrawn*

CB 101 LIPOPOLYSACCHARIDE-BINDING FACTOR IN BOVINE SERUM: INITIAL PURIFICATION. Philip N. Bochsler and Lajwanti S. Khemlani, Department of Pathobiology, The University of Tennessee, Knoxville, TN 37901

The response of inflammatory cells to bacterial lipopolysaccharide (LPS) may be regulated in part by factors that influence the cellular binding affinity or bioavailability of LPS. We have demonstrated the presence of LPS-binding factor(s) in bovine serum that complex with LPS and appear at a density of 1.35 g/cm^3 in CsCl gradients. Ion-exchange chromatography was used to fractionate bovine serum as an initial purification step. 40 ml serum with 5 mM EDTA was loaded onto the equilibrated column (Bio-Rex 70 resin; 41 mM NaCl in 50 mM phosphate buffer, pH 7.3 with 2 mM EDTA) and washed extensively. The column was subsequently washed with 220 mM NaCl (with phosphate buffer and EDTA), followed by a linear gradient (220 mM + 500 mM NaCl), and finally 1 M NaCl in phosphate buffer + EDTA. After dialysis and concentration, pools of collected fractions were tested for presence of LPS-binding activity (LPS-BAc) by: 1) the ability to enhance selective LPS-BAc of bovine serum (i.e. increased LPS-complex at 1.35 g/cm^3 in CsCl gradients), and 2) the ability to reconstitute LPS-BAc in depleted chromatographic fractions. A pooled chromatographic fraction (fraction 5) corresponding to the eluant from the 1 M NaCl wash had both of these properties. Increasing aliquots of fraction 5 (up to 200 μl) added to 300 μl of bovine serum quantitatively increased the LPS-complex at 1.35 g/cm^3 in CsCl gradients up to two-fold. Additionally, aliquots of fraction 5 added to LPS-BAc-depleted chromatographic fractions restored the presence of the LPS-complex at 1.35 g/cm^3. (Supported by USDA CSRS91-37204-6412 and the University of Tennessee Centers of Excellence.)

CB 102 INTERACTION OF SYNTHETIC ANALOGS OF LIPID A WITH MOUSE PRE-B LYMPHOCYTES, MATURE B LYMPHOCYTES, AND PERITONEAL MACROPHAGES. Richard Chaby*, Thierry Pedron☆, Robert Girard☆, Jacques Eustache#, Murty A.R.C. Bulusu#, Ingolf Macher#, Hermann Radzyner-Vyplel#, and Peter L. Stütz#, Université de Paris-Sud, Orsay, France*; Institut Pasteur, Paris, France☆; and Sandoz Research Institute, Vienna, Austria#.

Lipid A, the biologically active region of LPS, is known to accelerate the differenciation of pre-B lymphocytes to the mature stage, to induce mature B lymphocytes to enter into the cell cycle, and to bind to specific sites on the surface of macrophages with subsequent stimulation of these cells. To identify the structural elements required for these events, we used synthetic glucosamine-derived glycolipids, or synthetic lipopeptides, structurally related to Lipid A. Some of these lipids mimicked LPS for the induction of sIg expression on the 70Z/3 pre-B cell line. Neither phosphate groups, nor a glucosamine "backbone" were required for this activity, but the configuration of particular asymmetric carbons, and the distance between an anionic group and a N-acyl chain were critical parameters. A N,N'-diacylated and bisphosphorylated derivative of 2,3-dideoxy-2,3-diamino-D-glucose was a specific antagonist of LPS for this activity. The structural features required for the entry of mature B cells into the G1A phase of the cell cycle (cell enlargement) were identical to those necessary for stimulation of pre-B cells. On the other hand, there was no correlation between the potency of the lipids to interact with LPS-binding sites (inhibition of LPS-macrophage binding), and their ability to stimulate pre-B and mature B cells.

CB 103 BINDING OF [125I]LPS TO HUMAN MONOCYTES AND ITS INHIBITION BY SYNTHETIC LIPID A PARTIAL STRUCTURES. Werner Feist, Teruo Kirikae, Fumiko Kirikae, Shoichi Kusumoto*, Helmut Brade, Ulrich Schade, Ernst Th.Rietschel, Hans-Dieter Flad, and Artur J.Ulmer. Forschungsinstitut Borstel, Departments of Immunology and Cell Biology, and Immunochemistry and Biochemical Microbiology, D-2061 Borstel, Germany, *Department of Chemistry, Osaka University, Osaka, Japan.

Previously we have shown that the synthetic tetraacyl precursor Ia (compound 406 or LA-14-PP) was not able to induce monokine production in human monocytes but strongly antagonized LPS-induced formation of monokines. To analyze the molecular basis for this modulation, we have examined the binding of [^{125}I]-LPS to monocytes in the presence of synthetic lipid A and various partial structures (LA-15-PP, LA-22-PP, LA-14-PP, LA-24-PP, LA-23-PP). Compound 406 was found to inhibit the binding of [125I]LPS to human monocytes to the same degree as unlabeled LPS. When comparing the agonistic and antagonistic activity of lipid A and different partial structures at the functional level as well as at the level of binding, we found that these activities were dependent on the number, the nature and chain length of the fatty acids, and the number of phosphoryl groups. Unexpectedly, however, all synthetic lipid A partial structures tested were less potent in inhibiting the binding of [125I]-LPS than compound 406, even when possessing monokine-inducing capacity (like the hexaacyl E.coli lipid A, LA-15-PP). In summary, our data show that compound 406 inhibits LPS-induced monokine production at the level of binding of LPS to monocytes and furthermore, that high affinity binding of lipid A partial structures to monocytes is not necessarily associated with optimal induction of monokine production.

(Supported in part by Fonds der Chemischen Industrie [H.D.F., E.T.R.])

CB 104 TOXIC AND NON-TOXIC LPS MOLECULES COEXIST IN SOME ENDOTOXIN PREPARATIONS, AND BIND DIFFERENTLY TO THE HEPATIC TISSUE. Robert Girard#, Thierry Pedron#, and Richard Chaby*. Institut Pasteur, Paris, France#; and Université de Paris-Sud, Orsay, France*.

The chemical coupling of tyramine to LPS from different Enterobacteria and different chemotypes allowed the preparation of radiolabeled ^{125}I-LPSs with high specific activity (0.2 to 2x10^6 cpm/µg). The labeling procedure did not modify the toxicity of LPSs in galactosamine-sensitized mice, or their reactivity with specific immune-sera. One hour after iv injection of labeled LPS from *S.cholerae suis* (^{125}I-LPScs) to BALB/c nu$^+$ mice, 40% of the labeled LPS remained in the bloodstream and 40% was trapped in the liver. After re-isolation by phenol-water extraction, the hepatic fraction was as toxic as the initial LPS preparation, and induced in vivo tolerance to endotoxin, whereas the circulating fraction was considerably less toxic, and was unable to induce in vivo tolerance. It appears therefore that at least two different molecular species of LPS, with different clearance properties and in vivo effects, are present in the initial LPS preparation. A chromatography of the ^{125}I-LPScs preparation on a column of Aminohexyl-Sepharose 4B beads coated with glycine (by the glutaraldehyde method) and equilibrated in 0.1% Tween-20 in PBS, allowed the isolation of two fractions: a non-retained fraction with reduced toxicity (eluted with PBS/Tween-20), and a retained fraction with fully toxic activity (eluted with 5M NaCl in PBS/Tween-20). This result indicate that toxic and non-toxic molecular species of LPS are simultaneously present in some endotoxin preparations.

CB 105 BIOTIN-LPS CONJUGATES AS PROBES FOR LPS BINDING PROTEINS IN NORMAL AND ACUTE PHASE SERUM. M. Isla Halliday, Pauline J Erwin, Keith R Gardiner, Brian J Rowlands, Department of Surgery, The Queen's University of Belfast, Belfast BT12 6BJ, N.Ireland.

The role of serum proteins in the dissaggregation, detoxification and clearance of endotoxin has been under investigation for many years. To date, the identification and characterisation of LPS-binding proteins has been dependant on the use of radiolabelled-LPS probes in combination with density centrifugation techniques. Our aim was to prepare biotin-LPS probes which would facilitate in the identification of LPS-binding proteins in normal and acute phase serum.

Biotin-LPS conjugates were prepared by derivatisation of either the amino groups (biotin-aminocaproate-N-hydroxysuccinimide ester) or the glycosyl residues (periodate oxidation and biotin-hydrazide) of both rough and smooth LPS. Biological activity was assessed by the chromogenic LAL assay and the in vitro stimulation of blood peripheral monocytes to produce TNF. The ability of the probes to bind specific serum proteins was investigated using gradient pore gel electrophoresis, western blotting and the streptavidin detection system. All probes retained greater than 80 % of control activity. Incubation with normal serum identified a number of LPS binding proteins. Specificity was confirmed by preincubation with unlabelled LPS. Acute phase serum exposed additional proteins which require further investigation.

In conclusion, biologically active biotin-LPS probes can be prepared which are useful in the detection of serum LPS binding proteins. Additionally, exploitation of these probes in combination with streptavidin-agarose chromatography may led to simplified methodologies for the purification of putative binding proteins.

CB 106 ISOLATION AND CHEMICAL AND SEROLOGICAL CHARACTERIZATION OF THE PHOSPHORYLATED BACKBONE OF *ESCHERICHIA COLI* LIPID A, Otto Holst, Lore Brade, and Helmut Brade, Forschungsinstitut Borstel, Institut für Experimentelle Biologie und Medizin, D-2061 Borstel (F.R.G.)

We have described[1] lipid A antibodies reacting with lipid A or partial structures thereof with a distinct phosphorylation pattern. In addition, we showed that these antibodies bind to the hydrophilic backbone of lipid A and that acyl residues are not part of the epitope. Since fatty acids determine the three-dimensional conformation of lipid A depending on the physico-chemical environment, the deacylated phosphorylated lipid A backbone was isolated and converted into an artificial glycoconjugate allowing serological investigations in order to get direct proof for the above findings. Lipid A obtained from LPS of *Escherichia coli* F515 (Re mutant) was de-O-acylated by mild hydrazinolysis and then treated with strong alkali (4M KOH) to cleave the amide-linked fatty acids. After neutralization, the phosphorylated lipid A backbone was isolated by high-performance anion-exchange chromatography. Furthermore, its bisacetamido derivative was prepared by N-acetylation. Both compounds were structurally characterized by ^{1}H-, ^{13}C-, and ^{31}P-n.m.r. spectroscopy. The ligand was covalently linked to bovine serum albumine and used in serological assays. Several monoclonal antibodies were shown to bind to the fatty acid-free antigen. This proves unequivocally that fatty acids are not part of the lipid A epitopes recognized by the antibodies described.

1) Brade, L., Brandenburg, K., Kuhn, H.-M., Kusumoto, S., Macher, I., Rietschel, E. Th., and Brade, H., *Infect. Immun.*, 55 (1987) 2636-2644.

CB 107 X-RAY INVESTIGATIONS AND MOLECULAR MODELLING OF SAMONELLA TYPE S-FORM LPS, Manfred Kastowsky, Thomas Gutberlet and Hans Bradaczek, Freie Universität Berlin, Inst. f. Kristallographie, Takustraße 6, W-1000 Berlin 33, FRG.

Molecular modelling techniques have been applied to generate models of a complete *Salmonella* type bacterial S-form lipopolysaccharide consisting of the lipid A and core oligosaccharide portions and of four repeating units of the O-specific chain. Furthermore, X-ray diffraction experiments on dried samples of LPS with structures close to the calculated *Salmonella* model have been carried out and yielded molecular dimensions of LPS partial structures. Up to the Ra-structure calculated dimensions are in good agreement with experiments, and are: 2.4 nm for lipid A, 2.8 nm for Re- LPS, 3.5 nm for Rd-LPS and 4.4 nm for Ra-LPS. The maximum length of a stretched model carrying four repeating units was evaluated to be 9.6 nm, however, energetically preferred conformations showed a bent O-specific chain producing smaller extensions of about 5.0-5.5 nm. Monte Carlo simulations were carried out to study the flexibility within the oligosaccharide portion of the model. It was found that the Ra-structure is conformationally well defined, in contrast to the highly flexible O-antigenic chain. The Ra-structure appeared to have an approximate cylindrical shape. The O-specific chain , however, most likely does not adopt regular, i.e. helical conformations. Concerning the shape of the calculated models four regions can clearly be distinguished: lipid A, inner core, outer core and the O-specific chain. It is worth to note that we could not find any apparent separation within the inner core, i.e. between KDO and heptose regions. Rather, the inner core appeared to form one densely packed portion. Interestingly, the phosphate groups of lipid A and inner core were found to be exposed to the surface, whereas the KDO carboxyl groups were burried in the inside.

CB 108 INHIBITION OF LIPOPOLYSACCHARIDE (LPS)-BINDING TO J774.1 MOUSE MACROPHAGE-LIKE CELLS BY DEFINED LPS PARTIAL STRUCTURES.

Teruo Kirikae, F.Ulrich Schade, Fumiko Kirikae, Ulrich Zärhringer, Helmut Brade, Shoichi Kusumoto*, Tsuneo Kusama** and Ernst Th. Rietschel Forschungsinstitut Borstel, Institut für Experimentelle Biologie und Medizin, D-2061 Borstel, Germany and *Faculty of Science, Osaka University Osaka 560, **Daichi Pharmaceutical Co. Ltd., Tokyo, Japan

Natural and synthetic partial structures of LPS, including new synthetic compounds called PE-1 through PE-4 which contain an oxyethyl-linked (-O-CH2-CH2-) phosphoryl group in position 1, were examined in competitive binding experiments using ^{125}I-LPS (Re-mutant LPS) to clarify structural requirements of LPS for its binding to macrophages. S-LPS, Rb_2- and Re-LPS, synthetic *E.coli*-type lipid A (compound 506) and precursor Ia (compound 406) inhibited the binding of ^{125}I-LPS to macrophage-like J774.1 cells. Deacylated Re- and Rb2-LPS were completely inactive in inhibiting the binding of LPS. Among tetraacyl compounds, the inhibition-capacity of binding of LPS was in the order PE-4 (α-phosphorooxyethyl analog of 406) = 406 > > 404 (4'-monophosphoryl) > 405 (1-monophosphoryl). In hexaacyl compounds, 506, PE-1 (α-phosphorooxyethyl analog of 506) and PE-2 (differing from PE-1 in having C14:0 at C2 and C3 of ClcN) inhibited LPS-binding equally well, whereas PE-3 (differing from PE-2 in being not α- but β-phosphorooxyethyl) showed a substantial reduction in capacity of the binding-inhibition. Further, synthetic *Cromobacterium*-type lipid A (compound LA-22-PP) did not inhibit the binding. These results indicate that the presence of fatty acids and phosphoryl groups, especially in position 4', of lipid A are essential for binding of LPS to macrophages. The absence of the 3-hydroxyl groups within the acyl residues and linking of the phoshoryl residue through an oxyethyl group do not change the binding affinity to cell, whereas linking of the phosphoro-oxyethyl group in α- instead of β-anomeric configuration and the location of acyl groups are of importance for the binding to macrophages.

CB 109 GENERATION OF MONOCLONAL ANTIBODIES TO HUMAN LBP AND THEIR USE IN THE DETECTION OF LBP PROTEIN IN SERUM, *Didier Leturcq, Philip VanHook, Richard Smith, Peter Tobias*, Richard Ulevitch* and Ann Moriarty, The R.W. Johnson Pharmaceutical Research Institute, 3535 General Atomics Ct. # 100, San Diego, CA 92121, *The Scripps Research Institute, 10666 N. Torrey Pines Rd., La Jolla, CA92036*

Monoclonal antibodies have been generated to human LBP, purified from pooled, normal serum. Three different epitope groups have been characterized. One (8C9) of the three recognizes denatured LBP only. The other two monoclonal antibodies (1E8, 18G4) recognize native LBP. Monoclonal antibodies 1E8 and 18G4 both detect LBP when it is complexed with LPS; however, 1E8 is less efficient in the recognition of complexed LBP suggesting it may bind to the LPS binding site, or a region in close proximity, on LBP. Two of these monoclonal antibodies were used in the development of a sandwich ELISA for the detection of LBP in serum samples. Normal human serum samples were screened for the presence of LBP. Unlike the rabbit, in which circulating levels of LBP are in the ng/ml range prior to LPS stimulation, basal levels of LBP in normal human serum samples are in the low ug/ml range. Forty-four serum samples representing 29 different individuals were analyzed in the LBP detection assay. Ten individuals had serial bleeds of which all but two persons had similar LBP levels at later dates. In these two individuals the time span between the bleeds ranged from 2 to 5 months. The differences were only 2 to 3-fold higher than the previous time points. The LBP values for the 44 normal serum samples ranged from 1ug/ml to 24ug/ml, with an average of 7ug/ml. Serum samples from patients with different clinical states (i.e., burn, trauma, surgical, etc) are being evaluated for levels of LBP.

CB 110 MODULATION OF LPS-INDUCIBLE GENES BY SYNTHETIC LIPID X ANALOGUES, Carl L. Manthey,* Pin Yu Perera,* Peter L. Stütz,§ Thomas A. Hamilton,† and Stefanie N. Vogel,* *U. S. U. H. S., Bethesda, MD, †Research Institute, Cleveland Clinic Foundation Cleveland, OH, Sandoz Research Institute, Vienna, Austria.§

Lipid X derivatives have shown promise as potential non-toxic LPS mimetics retaining beneficial immunomodulatory activities and as LPS antagonists. Compound SDZ MRL 953 enhances nonspecific resistance to infection in murine models at doses 3 orders of magnitude greater than lipopolysaccharide (LPS), but is comparatively nontoxic. Compound 880.431 has been shown previously to be an LPS antagonist. We have examined by Northern blot analysis the ability of compounds SDZ MRL 953 and 880.431 to induce or inhibit, respectively, the expression of TNFα, IL-1β, and five other LPS-inducible genes, IP-10, D2, D3, D7 and D8, (Tannenbaum et al., J Immunol 140: 3640, 1988) in thioglycollate-elicited macrophages from C3H/OuJ mice. SDZ MRL 953 (0.1 - 10 μg/ml) induced all seven genes in C3H/OuJ macrophages. Gene induction was comparable to that induced by 0.1 - 10 ng/ml E. coli. K235 LPS, reflecting a roughly 3-log difference in potency. Compound 880.431 was unable to induce any of the genes detectably in C3H/OuJ macrophages, even at 100 μg/ml, but was able to markedly inhibit K235 LPS induction of gene expression in a dose-dependent fashion. Inhibition was specific, e.g., 880.431 did not block TNF expression by heat killed S. aureus, and inhibition could be completely overcome with high concentrations of LPS. These findings further define the LPS mimetic properties of SDZ MRL 953 and the antagonistic properties of 880.431. SDZ MRL 953 differed from LPS in potency, but induced a qualitatively similar pattern of gene expression. Compound 880.431 was shown to be devoid of agonist activity and able to inhibit uniformly the LPS induction of gene expression. These compounds are expected to prove useful as prototypes of therapeutics designed to boost the immune response or block septic shock and to serve as useful reagents for the further characterization of LPS receptor proteins. [NIH AI18797 (SV) and CA39621 (TH)]

CB 111 RECOGNITION OF ENDOTOXIN AND SERUM-RESISTANT BACTERIA IN WHOLE BLOOD BY BPI_{23}, A RECOMBINANT 23 KD LPS-BINDING FRAGMENT OF BACTERICIDAL PERMEABILITY INCREASING PROTEIN (BPI), K. Mészáros, P. Conlon, H. Gazzano-Santoro, L. Grinna, A. Horwitz, B. Parent, T. Parsons, G. Theofan and P. Trown, XOMA Corporation, Berkeley, CA 94710. P. Elsbach and J. Weiss, New York University School of Medicine, New York 10016.

BPI is an LPS-binding protein with potent bactericidal and endotoxin-neutralizing activity that is found in the azurophilic granules of neutrophils. A recombinant protein, BPI_{23}, which corresponds to the N-terminal half of human BPI, has been shown to exhibit similar *in vitro* biological activity to the 55 kD parent molecule and to contain the LPS-binding site. These properties suggest that BPI_{23} has potential as a therapeutic agent to treat Gram-negative sepsis. Since it is known that various serum proteins can bind LPS, it is important to determine if BPI_{23} retains its potent bactericidal and anti-endotoxin activity in the complex environment of whole blood. To this end, the binding of ^{125}I-labeled BPI_{23}, dissolved in whole human blood, to lipid A-coated, or uncoated, plastic wells following incubation for 1 hr at 37°C was determined. Specific, saturable binding of ^{125}I-BPI_{23} in whole human blood to immobilized lipid A was demonstrable in these studies. The bactericidal properties of BPI_{23} were also preserved, since the survival of serum-resistant K1-encapsulated *E. coli* in whole blood was significantly reduced (>99%) by low doses of BPI_{23} (0.25 ug/ml). To evaluate the endotoxin-neutralizing activity of BPI_{23} in whole blood, we used a model system based on the activation of monocytes and PMNs by LPS resulting in chemiluminescence due to the release of active oxygen metabolites. BPI_{23} at low doses inhibited by greater than 90% the chemiluminescence induced in whole human blood by *E. coli* O113 LPS. We have recently reported that another cellular response, the LPS-induced accumulation of TNF in whole blood, is inhibited by a BPI_{25}, a proteolytic fragment of intact BPI that is slightly larger than recombinant BPI_{23} (J. Exp. Med. 174:649, 1991). These observations demonstrate that, in whole blood, BPI_{23} recognizes lipid A and retains its potent bactericidal and endotoxin-neutralizing activity.

CB 112 DETAILED COMPOSITION OF NATURAL MPL® IMMUNOSTIMULANT, Kent R. Myers and D. Scott Snyder, Ribi ImmunoChem Research, Inc., Hamilton, MT 59840. MPL® immunostimulant is an attenuated endotoxin derivative prepared by sequential acid and alkaline hydrolyses of *S. minnesota* R595 lipopolysaccharide (LPS). These treatments reduce greatly the toxic attributes of the parent LPS, while retaining a significant degree of immuno-stimulating activity. Accordingly, MPL® immunostimulant is being considered as a prophylactic treatment for prevention of septic shock and as a vaccine adjuvant. Analysis of MPL® immunostimulant by TLC and HPLC reveals that it is a complex mixture of species. The present study was undertaken to more fully characterize this material with respect to composition. A production lot of MPL® immunostimulant (Lot 039-179) was fractionated by preparative TLC into the 3 major TLC bands resolvable on silica gel G with $CHCl_3$:MeOH:H_2O:NH_4OH 50:31:6:2 (v/v): TLC 3, R_f=0.47; TLC 5, R_f=0.41, and; TLC 7, R_f=0.36. Each TLC fraction was then methylated and analyzed by reverse-phase HPLC. TLC 3 contained one major and one minor component, TLC 5 contained 3 major and several minor components, and TLC 7 contained 2 major and several minor components. The 6 major species were isolated in pure form and analyzed by fast-atom bombardment mass spectrometry (FAB-MS), which allowed observation of both the parent molecular ion and the non-reducing end oxonium ion fragment for each compound. This information, combined with knowledge of the structure of *S. minnesota* lipid A, allowed structures to be postulated for each of the isolated species. It was found that the TLC 3 component contained 6 fatty acyl groups, the 3 species in TLC 5 contained 5 groups, and the two TLC 7 species contained 4 groups. These structures were found to contain several common features: 1) a ß-1',6-diglucosamine backbone, monophosphorylated on the non-reducing end; 2) fatty acyl groups at the 2-, 2'-, and 3'-positions, with ß-myristoxymyristoyl always present at the 2'-position, and; 3) a free hydroxyl group at the 3 position. The species differed with respect to 1) ß-hydroxymyristoyl or ß-palmitoxymyristoyl at the 2-position and 2) ß-hydroxymyristoyl, ß-myristoxymyristoyl, or myristenoyl at the 3'-position. This heterogeneity can be attributed to fatty ester hydrolysis and elimination occurring during the acid and alkaline treatments.

CB 113 LIPOPOLYSACCHARIDE-BINDING PROTEINS OF LIMULUS AMEBOCYTE LYSATE, Robert I. Roth, Peter S. Tobias, and Jack Levin, Depart. of Laboratory Medicine, Univ. California Sch. Medicine, San Francisco, CA 94121, and Scripps Clinic and Research Foundation, La Jolla, CA, 92037.

Limulus amebocyte lysate, obtained from the blood cells of the horseshoe crab (Limulus polyphemus), contains a coagulation system which is activated by bacterial lipopolysaccharide (endotoxin, LPS). The interaction of LPS with a LPS-sensitive coagulation protein is believed to be the initial event in coagulation. We utilized a photoactivatable, cleavable, ^{125}I-labeled derivative of S. minnesota LPS (LPS-ASD) to identify LPS binding proteins. A chromatographic fraction of Limulus lysate which contained the endotoxin sensitive factor(s) of the coagulation cascade was studied. The lysate fraction was incubated with LPS-ASD, the probe was activated with UV light, and the mixture was electrophoresed in polyacrylamide gels in the presence of SDS and 2-ME. LPS binding proteins were identified by autoradiography of the gels, and binding was judged to be specific by inhibition of radioactive bands by cold, underivatized LPS or purified Lipid A. Many major proteins in the lysate fraction did not bind the probe, substantiating that proteins designated as LPS-binding had true affinity for LPS. An 82kDa protein, a major protein component of this fraction from Limulus lysate, was identified as a specific LPS binding protein. Incubation of whole Limulus lysate with antiserum to this protein resulted in marked enhancement of sensitivity of lysate to subsequently added LPS, suggesting that this protein is a negative regulator of coagulation as the result of its ability to bind LPS. A minor 50kDa protein component of lysate also was identified as a specific LPS binding protein, and is a candidate for the LPS-sensitive coagulation protein in Limulus. Neither LPS binding protein apparently has been described previously in Limulus amebocyte lysate.

CB 114 EXPRESSION OF RECOMBINANT HUMAN LBP IN E.COLI AND IN A BACULOVIRUS SYSTEM

Ralf R. Schumann (1), Loren Hatlen, Peter S. Tobias, John C. Mathison and Richard J. Ulevitch,
Department of Immunology, The Scripps Research Institute, La Jolla, Ca., 92037 (1): present address: Dep. of Internal Medicine, University of Freiburg, D-7800, FRG.

cDNA of human LPS Binding Protein was constructed into two different vectors: A pBR 322 derived vector utilizing the SP2 promoter specific for transformation of E.coli and a pVL 1392 vector that was used to coinfect Sf-9 insect cells with the baculovirus. High levels of intracellular expression of a 50 kD protein were achieved in the bacterial system. A protein band, visible by Coomassie-staining of an SDS-PAGE of the solubilized transformed cells, which was not seen in the untransformed cells, was immunoreactive with anti-LBP antibodies in a western-blot. Perhaps due to a high level of expression, refractile bodies containing the expressed protein were formed, and could be solubilized only by a protocol including sonication and 6M Guanidine treatment. The solubilized recombinant LBP was still immunoreactive in a western blot, but did not exhibit any LPS binding. The baculovirus system yielded expression and secretion of a 55 kD protein that was immunoreactive with anti-LBP antibodies in a western-blot and that expressed biological activiy after purification. 11 l of cultur supernatant yielded 10,25 mg protein after purification via ion-exchange chromatograohy with Bio-Rex 70 and Mono-Q resins. The purified protein bound to LPS immobilized on microtiterplates and enhanced LPS induced TNF-production in rabbit peritoneal elicited macrophages, in a manner comparable to purified native LBP. As the size of the glycosylated native protein is 58/60 kD, we assume that the baculovirus-derived protein has an incomplete glycosylation pattern but still might be a helpful tool in studying structure function relationships in LBP.

CB 115 MOLECULAR CLONING OF RAT LIPOPOLYSACCHARIDE BINDING PROTEIN. Grace L. Su, Stewart C. Wang, David Geller, Qi Wang, Richard L. Simmons, Timothy R. Billiar, David J. Tweardy. Departments of Medicine, Surgery, Pathology and Molecular Genetics and Biochemistry of the University of Pittsburgh School of Medicine, PA, 15261.

LBP is an acute phase protein which appears to play a major regulatory role in the *in vivo* response to endotoxin. The cDNA clones for rabbit and human LBP have previously been characterized and reported by Ulevitch. Since the rat is the basis for several important *in vivo* models of the immune response to sepsis and injury, we have sought to clone the cDNA for rat LBP in order to examine its function and regulation in the animal model. Total RNA was isolated from human liver using guanidinium isothiocyanate-cesium chloride gradient ultracentrifugation. Reverse Transcription-PCR was then performed using primers designed from the published sequence of human LBP. The identity of the resultant amplification product was confirmed using restriction analysis and purified. This purified PCR product containing human LBP sequences was labeled by random priming and used to probe 500,000 plaques of an amplified lambda phage library constructed from poly-A RNA isolated from the liver of rats previously injected with *C. Parvum*. 20 phage clones remained positive after three rounds of screening. The longest positive clone was 600 base pairs in length. Sequencing of three clones revealed approximately 79% nucleotide homology with human LBP. Use of one of the cloned inserts (pGS1) for Northern blot analysis revealed a band of 2.3 Kb that is strikingly upregulated in hepatocytes isolated from livers of *C. Parvum* injected rats when compared to hepatocytes from untreated animals.

Isolation of a full length cDNA for rat LBP will enable us to perform structure-function studies in rat models of sepsis as well as examine the regulation of LBP production at the gene level.

CB 116 INDUCTION AND MODULATION OF MONOKINE PRODUCTION BY PHOSPHONOOXYETHYL ANALOGUES OF LIPID A. Artur J.Ulmer, Holger Heine, Werner Feist, Shoichi Kusumoto*, Tsuneo Kusama§, Teruo Kirikae, Fumiko Kirikae, Helmut Brade, Ulrich Schade, Ernst Th. Rietschel, and Hans-Dieter Flad. Forschungsinstitut Borstel, Departments of Immunology and Cell Biology, and Immunochemistry and Biochemical Microbiology, D-2061 Borstel, Germany, *Department of Chemistry, Osaka University, Osaka, Japan, §Daichi Pharmaceutical Co. Ltd., Tokyo, Japan.

We have analyzed the biological activity of four new synthetic partial structures of lipid A, termed PE-1, PE-2, PE-3, and PE-4. All structures contain an α-oxyethyl-linked (-O-CH_2-CH_2-) phosphoryl group in position 1 of the reducing glucosaminyl residue (GlcN I) of lipid A. PE-1 is a hexaacylated analogue of E.coli lipid A (compound 506). PE-2 differs from PE-1 in having nonhydroxylated fatty acids at GlcN I. In PE-3, GlcN I is present in the ß-anomeric form, and PE-4 is an analogue of tetraacyl precursor Ia (compound 406). When determining TNF, IL-1, and IL-6 release by human mononuclear cells (MNC), we found that PE-1, PE-2 and PE-3 induced monokine production, whereas PE-4 did not. The activity of PE-1 was similar to that of compound 506. PE-2 showed a somewhat weaker effect than PE-1. Compound PE-3, on the other hand, was substantially less active than compound PE-1 and PE-2. Furthermore, we tested whether PE-4 had the same inhibitory effect on LPS-induced monokine production as compound 406. We found that indeed PE-4 is, like compound 406, an antagonist of LPS in inducing monokine production. Competitive binding experiments using [125I]LPS indicated that PE-4 performs its inhibitory activity at the level of binding of LPS to monocytes. In summary, these results show that attachment of the glycosidic phoshoryl residue via an oxyethyl group had no effect on the biological activity of lipid A or related partial structures. The absence of the 3-hydroxyl groups within the acyl residues at GlcN I had only a small effect on biological activity. On the other hand, the anomeric configuration of GlcN I was found to be of great biological relevance as the α-anomer expressed high, the ß-anomer low endotoxic activity.

(Supported in part by Fonds der Chemischen Industrie [H.D.F., E.T.R.])

LPS Receptors and CD14

CB 200 STIMULATION OF TNF PRODUCTION BY URONIC ACID POLYMERS AND LPS: THE INVOLVEMENT OF CD14, Terje Espevik, Marit Otterlei, Anders Sundan, Samuel D. Wright*, Gudmund Skjåk-Bræk and Olav Smidsrød, Institute of Cancer Research and Institute of Biotechnology, University of Trondheim, N-7005 Trondheim, Norway, and *Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, New York 10021.

Polymers of ß1-4 linked uronic acids constitute a family of polysaccharides with cytokine stimulatory activity (Otterlei et al. J.Immunotherapy 10:286, 1991). The mechanisms behind the cytokine stimulatory activity of uronic acid polymers are not known. CD14 has been implicated as a functional receptor for the LPS/LBP complex on monocytes (Wright et al., Science, 249:1431, 1990). In this study binding characteristics as well as biological activity of uronic acid polymers and LPS have been compared. Our data indicate that poly mannuronic acid (poly M) binds to CD14 in the presence of human serum and that CD14 is involved in poly uronic acid induced TNF production in monocytes. LPS induces the CD14 negative U373 cell line to produce IL-6. However, poly M did not bind to U373 cells and did not induce IL-6 production in these cells. In conclusion, these results indicate that uronic acid polymers induce TNF production through mechanisms which involve CD14, whereas LPS may stimulate cells also through other mechanisms than mediated by CD14.

CB 201 SOLUBLE CD14 INHIBITS THE ACTIVATION OF MONOCYTES INDUCED BY LPS, Alain Haziot, Guo Wei Rong, Ben Z. Tseuberi and Sanna M. Goyert, Division of Molecular Medicine, North Shore University Hospital / Cornell University Medical College, Manhasset, New York 11030.

CD14 is a PI-anchored glycoprotein expressed primarily on the surface of monocytes. CD14 has recently been shown to be a receptor for LPS when LPS is complexed to an acute phase protein, LBP (LPS binding protein). Binding of the LPS:LBP complex to monocytes via CD14 *in vitro* causes them to be activated and to release large amounts of cytotoxic factors including TNF. This pathway of activation, resulting in the release of TNF, is thought to be the major pathway leading to endotoxin shock; therefore, inhibition of this pathway of activation might provide a useful therapy. One potential method for inhibiting LPS:LBP induced monocyte activation would be to supply soluble CD14 which could bind to the active site of the LPS:LBP complex in solution and therefore reduce the concentration of the activating component and prevent the response. In order to test this hypothesis, native and recombinant forms of soluble CD14 were isolated from the urine of a patient with nephrotic syndrome and from Baculovirus cultures, respectively. *In vitro* studies show that both forms of soluble CD14 can bind LPS:LBP complexes in solution and inhibit the secretion of TNF by monocytes stimulated with LPS:LBP. These results suggest that soluble CD14 can regulate the activation of monocytes by LPS and provide a basis for potential therapy.

CB 202 COMPETITION BETWEEN LPS-BINDING PROTEIN (LBP) AND ANTI-LPS ANTIBODY IN LPS-INDUCED TNF SECRETION OF HUMAN MONOCYTES (MO). D. Heumann[1], P. Gallay[1], C. Barras[1], R.J. Ulevitch[2], P.S. Tobias[2], M.P. Glauser[1] and J.D. Baumgartner[1]. [1]Division of Infectious Diseases, CHUV, Lausanne, Switzerland and [2]Department of Immunology, Scripps Clinics, La Jolla, CA 92037.

It has been shown that anti-LPS antibodies (Ab) increase LPS clearance by the reticulo-endothelial system, including Mo, and suppress TNF secretion in animals challenged with LPS. In vitro, LPS reacts with LBP contained in serum, inducing a CD14-mediated TNF secretion by Mo. To determine the mechanism by which anti-LPS Ab suppresses TNF production in vivo, we studied in vitro the respective effects of anti-LPS Ab and of LBP 1) on the binding of fluorescent (FITC) O111 LPS on Mo using FACS analysis and 2) on TNF production. We found that the addition of LBP-containing serum to cultures of Mo induced a strong binding of LPS to Mo and induced TNF secretion, both of which could be inhibited by an anti-CD14 mAb (MY4). In contrast, when an anti-O111 LPS mAb (D6B3) was added to serum, most of the LPS binding could no longer be inhibited by MY4, suggesting that binding occured to receptors other than CD14. The binding pattern was different in the presence or in the absence of complement, suggesting a binding of LPS-anti-LPS immune complexes through Fc or complement receptors. When the effect of anti-LPS Ab on TNF secretion by Mo in the presence of LBP-containing serum was measured, we found that D6B3 totally prevented TNF secretion induced by 0.1 ng/ml of LPS, but only partially by higher concentrations. The TNF secretion occuring despite D6B3 was due to a triggering of CD14 since it could be suppressed by MY4. In conclusion, anti-LPS Ab mediate the binding of LPS to Fc or complement receptors, thus competing with the binding of LPS to CD14, and therefore decreasing TNF production by Mo. LPS-mediated TNF production was suppressed at LPS concentrations that are likely to occur in vivo.

CB 203 SYNTHESIS, PROCESSING AND RELEASE OF THE CD14 ANTIGEN BY CALCITRIOL INDUCED HL-60 CELLS, William S. Kloetzer, Karen Kabat-Stegman and Donald E. Wegemer. The R.W. Johnson Pharmaceutical Research Institute, San Diego, CA, 92121.

The mature CD14 antigen, a receptor for the LPS/LBP serum protein complex, is a 55kd phosphatidylinositol glycan-linked protein expressed primarily on cells of the monocytic lineage. We show that monoclonal antibody (MAb) immunoprecipitation (IP) of [^{35}S]CD14 antigen from calcitriol induced HL-60 cells detects a 50kd precursor which is processed to the 55kd form. Rabbit antiserum against a C term-derived synthetic 15-mer detects the 50kd but not the processed 55kd species. Both proteins incorporate [^{3}H]glucosamine but only the larger one incorporates [^{3}H]ethanolamine. Immune complex exposure to N-glycosidase F (NGF) reduces the apparent MW of both [^{35}S]antigens but does not eliminate the relative mobility (RM) shift associated with antigen processing. A mixture of NGF and neuraminidase (NA) blocks the RM shift which indicates that attachment of sialic acid accompanies precursor maturation. CD14 antigens, labeled in the presence of glycosylation inhibitor tunicamycin B_2, display lower MWs that still undergo RM shifts concurrent with C term processing. We conclude that protein glycosylation accounts for most of the apparent MW changes that accompany CD14 antigen processing.

IP from 24hr conditioned growth medium (CGM) of [^{35}S]cells shows a diffusely migrating antigen of about 53kd. Soluble antigen can be detected from [^{35}S]met and [^{3}H]glucosamine but not [^{3}H]ethanolamine labeled cells. Two forms of sequentially released CD14 are resolved by NGF and NGF/NA treatment of immunoprecipitates and by IP from tunicamycin B_2 exposed cells. Antigen sequentially collected from 30min - 3hr CGM is detected by the C term 15-mer antiserum whereas the 3hr - 24hr released species is not. We conclude that a nearly full-length species of CD14 antigen escapes the peptidase-phosphatidylinositol glycan tailing mechanism; it is modified with sialic acid and rapidly released from the cell. A smaller species, derived by alternate processing or proteolytic release of surface antigen, is truncated at the C terminus prior to release.

A possible regulatory influence of spontaneously released CD14 antigens on LPS activated TNFa release was tested. LPS concs as low as 10pg/ml stimulate TNFa release from indomethacin-treated DHvD$_3$/HL-60 cells in growth medium containing 10% human AB serum. The presence of 24hr CGM, compared to fresh growth medium changed immediately prior to LPS addition, has little or no effect on TNFa release. We conclude that neither form of spontaneously released CD14 antigen actively participates in regulating LPS induction of TNFa release.

CB 204 SOLUBLE CD14 RELEASE IN HUMAN MONOCYTES AND MACROPHAGES, REGULATION BY INTERFERON-GAMMA AND INTERLEUKIN-4, Regine Landmann, Ada Fisscher and Jean-Paul Obrecht, Lab. Infectious Diseases, Department of Research, University Hospital, Basel, Switzerland

The myeloid cell molecule CD14 exists in a phosphatidylinositol-anchored membrane bound (mCD14) and two soluble forms (sCD14). mCD14 is engaged in LPS mediated TNFa production and in delivery of a negative signal to activated T cells. The function and regulation of sCD14 are unknown. The present study investigates the release of sCD14 in cultures of mononuclear leucocytes (PBL), elutriated monocytes and monocyte derived macrophages. sCD14 was measured by ELISA (recognizing both forms) in the supernatant of cells cultured for 15 and 45 h in the absence or presence of selected cytokines. sCD14 release occurred constitutively and correlated with cell number, the amount produced within 15h was lower in macrophages than in monocytes. In monocytes differentiating into macrophages either with AB serum or with 1,25OH-Vit.D3 instead of serum, cumulative release was linear from day 1 to day 7. Cycloheximide and Actinomycin D inhibited sCD14 production. rIFNg and IL-4 directly decreased sCD14 release in monocytes and macrophages. rIL-2 and rIFNa reduced sCD14 release into the supernatants of PBL only. Use of anti IFNg antibodies indicated that the downregulation of sCD14 release by rIL-2 and rIFNa was partially (rIFNa) or completely (rIL-2) due to induction of endogenous IFNg. The downregulation of sCD14 release by all four cytokines was both time and dose dependant. rIFNg and sCD14 added simultaneously exerted a synergistic effect on sCD14 downregulation. In conclusion, sCD14 release may have an immunomodulatory role in monocytes, is apparently not related to macrophage differentiation, and is selectively downregulated during an immune response when levels of IFNg and IL-4 are high.

CB 205 EXPRESSION OF CD14 DRAMATICALLY INCREASES SENSITIVITY TO LPS IN 70Z/3 CELLS. J.-D. Lee[1], K. Kato[1], P.S. Tobias[1], T. Kirkland[2] and R.J. Ulevitch[1]. [1]The Scripps Research Institute, La Jolla, CA; [2]University of California, San Diego, San Diego, CA.

While it is generally agreed that LPS stimulates cells by receptor dependent mechanisms the identity of such receptors is still not certain. Recent data supports the contention that in cells of myeloid origin the cell membrane protein CD14 serves as a receptor for complexes of LPS and LPS binding protein (LBP) and plays an important role in regulating LPS-dependent cell stimulation. However, many cell types lack CD14, i.e., murine B-cells, endothelial cells, fibroblasts, etc. but nevertheless respond to LPS via receptor dependent mechanisms. Thus there may be CD14 dependent and independent pathways for LPS stimulation of cells. Because these pathways appear to be linked in myeloid cells we hypothesized that introduction of CD14 into CD14-negative, LPS responsive cells might substantially enhance the response to LPS. To do this we have engineered stable transfectants of the murine pre-B cell 70Z/3 that express rabbit CD14. Parental or vector transfected control cells were devoid of CD14 mRNA while 70Z/3 cells transfected with rabbit CD14 cDNA cloned into pRc/RSV were found to have CD14 mRNA. Cell surface CD14 on transfected cells was detected by FACS. Cell stimulation was measured by quantifying surface IgM expression 24 hrs after stimulation with LPS, complexes of LPS and LBP, or interferon-gamma (IFN) in three groups of 70Z/3 cells; (i) parental (70Z/3), (ii) vector-transfected (70Z/3-RSV), and (iii) CD14-transfected (70Z/3-CD14$^+$). Stimulation with IFN was identical in all three groups. In marked contrast 70Z/3-CD14$^+$ were up to 1000-fold more sensitive to LPS or LPS-LBP complexes when compared to 70Z/3 or 70Z/3-RSV cells. The effect was noted with R- or S-form LPS and with synthetic lipid A. These data suggest that the CD14-dependent and independent pathways for LPS-stimulation may be linked and support the contention that the LPS receptor on myeloid cells consists of at least two separate proteins, CD14 and an as yet unidentified additional membrane protein.

CB 206 EXPRESSION OF A SOLUBLE FORM OF CD14 AND THE DEMONSTRATION OF ITS BINDING TO LPS:LBP COMPLEXES, *Ann Moriarty, Jane Gao, Juli DeGraw, Philip VanHook, Ashok Kumar & Didier Leturcq, The R.W. Johnson Pharmaceutical Research Institute, 3535 General Atomics Ct. SanDiego,CA92121*

The receptor for LPS:LBP complexes has been identified as CD14 (Wright et al, 1990). CD14, a GPI-linked protein present on the surface of mature monocytes and macrophages, has both a cell-associated and a spontaneously released form. Both forms are recognized by all anti-CD14 monoclonal antibodies thus far tested. We have expressed, by recombinant DNA methods, two different clones of the CD14 protein; one full-length and the other truncated at the C-terminus. Utilizing a clone of CD14, provided by B. Seed (Blood, 73:284, 1989), a full-length and truncated DNA fragment were inserted into the expression vector pEE14 (Celltech). Two clones, identified as 523 and 847 for the full-length and truncated versions, respectively, expressed high levels of soluble CD14 in transfected CHO cells. Affinity purified, recombinant protein was isolated and evaluated for the ability to bind LPS:LBP complexes. The complexes were formed on a solid phase by the initial addition of LBP followed by LPS or preformed and captured with an anti-LBP monoclonal antibody which recognizes LBP:LPS complexes. Soluble CD14 from both 523 and 847 was capable of binding LPS:LBP complexes, with clone 523 exhibiting the greater degree of binding. N-terminal sequencing of both proteins resulted in the predicted TTPEPCE amino acids residues. C-terminal sequence analysis of the two sCD14 proteins will be utilized to determine how each protein is processed to the soluble form. The generation of large quantities of native, spontaneously released soluble CD14 is being pursued to compare the binding properties of this form versus the recombinant-derived proteins. The potential of soluble CD14 as a therapeutic for septic shock is being evaluated.

CB 207 IDENTIFICATION OF SPECIFIC LPS BINDING PROTEINS ON HUMAN PERIPHERAL BLOOD MONOCYTES. Ronald L. Rabin, Marcia M. Bieber, and Nelson N.H. Teng. Department of Gynecology and Obstetrics, Stanford University School of Medicine, Stanford, CA 94305

We used the radioiodinated photoactivatable crosslinker sulfosuccinimidyl 2-(p-azidosalicylamido)ethyl-1,3'-dithiopropionate (SASD) to identify specific receptors for LPS on the human peripheral blood monocyte (PBM). After binding and crosslinking, the monocytes were solubilized in a Triton X-100 buffer, and the resulting membrane and cytoplasmic proteins were analyzed with two dimensional electrophoresis polyacrylamide gels and autoradiography. We labelled with ^{125}I both the wild type E. coli O111:B4, and its rough strain mutant J5 LPS chemotypes, and found that both chemotypes displayed the same pattern of binding, thus eliminating, in this model, a role for polysaccharide chain mediated receptors. We found one major, inhibitable binding site on human PBMs at 72 kD, pI 5.95. That this a membrane protein was confirmed by specific membrane extraction with an n-octyl ß-D-glucopyranoside (2OG) detergent preparation. Binding was concentration and temperature dependent, enhanced in the presence of calcium and magnesium, and inhibitable by excess unlabelled smooth or rough LPS. The 72 kD binding site resembles the 73 kD receptor described by Lei and Morrison. Both consist of three binding sites, all of the same MW, but differing slightly in pI. There was a quantitative difference between binding of ^{125}I-ASD-J5 and ^{125}I-ASD- O111:B4, the former binding >2 fold than the latter. Experiments examining inhibition by soluble peptidoglycan are now in progress.

CB 208 MACROPHAGES FROM ENDOTOXIN-TOLERANT MICE PRODUCE AN INHIBITOR OF THE SYNTHESIS OF TUMOR NECROSIS FACTOR IN NORMAL MACROPHAGES, F. Ulrich Schade, Sylvia Düpow, Claudia Franke and Ernst Th. Rietschel. Forschungsinstitut Borstel, Parkallee 22, W-2061 Borstel, FRG.

Supernatants of peritoneal macrophages, isolated from endotoxin (ET)-tolerant mice and treated with ET *in vitro* were found to elaborate an activity which inhibited the synthesis of tumor necrosis factor (TNF) by peritoneal macrophages of normal mice. The inhibitory activity was present after treatment with ET in vitro and was not detected in unstimulated cells from tolerant mice or "tolerant" cells treated with phorbol myristate acetate (PMA) and γ-interferon (γ-IFN). Stimulation of macrophages from normal mice, either with ET or PMA/γ-IFN did not result in the expression of the inhibitory activity. On the other hand, the inhibitory activity did not interfere with the cytotoxic activity of recombinant mouse TNF or TNF-containing mouse serum towards TNF-sensitive WEHI cells. Moreover, the inhibitory activity in the supernatants of macrophages from tolerant mice extended to PMA/γ-IFN-induced synthesis of TNF. The inhibitory activity was termed "TNF inhibitor of macrophages" (TIM). TIM was purified by ultrafiltration, gel-permeation and anion exchanger chromatography. SDS-PAGE of the active fraction showed a single band after transfer to nitrocellulose and staining with amidoblack or an antiserum obtained in rabbits which neutralized TIM activity. Our data suggest that TIM is a physiological inhibitor of TNF formation and may play a role in endotoxin tolerance.

Cell Activation

CB 300 REGULATION OF PAF RECEPTOR-MEDIATED PROSTAGLANDIN E_2 FORMATION IN LPS PRIMED P388D_1 MACROPHAGE-LIKE CELLS, R. Asmis and E. A. Dennis, Department of Chemistry, University of California at San Diego, La Jolla, CA 92093-0601.

The macrophage-like P388D_1 cells can be stimulated by Ca^{2+} ionophore A23187 and by platelet activating factor (PAF) in a receptor-mediated manner to produce arachidonic acid metabolites such as PGE_2 [Lister et al. (1989) J. Biol. Chem. *264*, 8520 - 8528]. The PAF stimulated PGE_2 release is increased 3-5 fold when the P388D_1 cells are primed with bacterial lipopolysaccharide (LPS) [Glaser et al. (1990) J. Biol. Chem. *265*, 8658 - 8664]. We have now found that LPS primed PAF stimulation is inhibited by BAPTA and TMB-8 in a dose-dependent manner suggesting that a rise in intracellular Ca^{2+} is required for PGE_2 formation. EGTA also inhibited PGE_2 formation; however, La^{3+} had no effect. Therefore, it remains unclear if an influx of extracellular calcium is required for PAF induced PGE_2 formation. PAF was also found to induce the release of IP_3 from phosphoinositides. The release of IP_3 and the formation of PGE_2 both are inhibited when the cells are pretreated with pertussis toxin. LPS and Ionomycin, however, were unable to increase IP_3 levels. Genistein, an inhibitor of tyrosine-specific protein kinase, was also unable to inhibit PAF induced increase in IP_3 levels. These results suggest that PAF activates a phospholipase C (PLC) via a pertussis toxin sensitive G-protein to release IP_3, but this PLC does not seem to be activated by tyrosine phosphorylation. It has been well demonstrated that the increase in IP_3 levels causes the release of intracellular calcium. This signaling pathway is independent of LPS priming. Finally, we have studied the effect of PGE_2, one of the products of P388D_1 cell stimulation, and have demonstrated that this prostaglandin increases cAMP levels in P388D_1 cells at nM concentrations. LPS, PAF, and forskolin had no effect on cAMP levels; however, Cholera toxin was a very potent activator of cAMP formation. These results suggest that PGE_2 activates adenylate cyclase in a receptor-mediated manner via a cholera toxin sensitive G-protein. The role of cAMP in our system is not clear yet, but this new signaling pathway could be part of a feedback mechanism in P388D_1 cells.

CB 301 IDENTIFICATION OF REGULATORY ELEMENTS IN THE UNTRANSLATED REGIONS OF TUMOR THE NECROSIS FACTOR (TNF) GENE

Flavia Bazzoni, Veronique Kruys, Alexander Shakhov*, Victor Jongeneel* and Bruce Beutler, Howard Hughes Medical Institute, Dallas TX 75235-9050, *Ludwig Insitute for Cancer Research, Epalinges, Switzerland.

TNF gene expression in extrahematopoietic cells of the L-929, Hela and NIH 3T3 lines is essentially governed by the 3' untranslated region (UTR). Reporter constructs reveal that the TNF promoter is constitutively utilized in each of these cell types, and that expression can be suppressed by the presence of the TNF 3' UTR. However, in L-929 cells, the TNF 3' UTR fails to completely abolish expression of the reporter gene, provided that the 5' UTR is also included in the construct. Escape from the suppressive effect of the 3' UTR results from the action of a trans-dominant factor. In order to determine which elements were responsible for suppression and for escape, deletion constructs were synthesized and analyzed for expression in transient transfection assays. Deletions were first introduced in the 3' UTR. Analysis of the expression of these deletion constructs in Hela cells and in NIH 3T3 cells revealed that the main suppressive element is the conserved UA-rich sequence. In order to elucidate the mechanism by which the 5' UTR overcomes the suppressive effect of the 3' UTR in L-929 cells, deletion analysis of the 5' UTR was also performed. The highest expression of the reporter gene is obtained with a construct containing the sequence between the 40th and the 80th nucleotides of the TNF 5' UTR. It is known that the TNF 3' UTR plays an essential role in LPS-induced TNF gene expression in macrophages. Since both 3' and 5' UTRs influence TNF biosynthesis in extrahematopoietic cells, the role of the 5' UTR in modulation of TNF gene expression in LPS-induced macrophages is under investigation.

CB 302 EFFECT OF ENDOTOXIN (LPS) TOLERANCE ON MACROPHAGE (MØ) EICOSANOID PRODUCTION; POTENTIAL MECHANISMS OF DESENSITIZATION, James A. Cook, Keith A. Coffee, W.C. Wise and P.V. Halushka, Medical University of South Carolina, Charleston, SC, 29425

Guanine nucleotide regulatory (G) proteins mediate membrane agonist-receptor coupling mechanisms and may mediate LPS- stimulated rat peritoneal MØ arachidonic acid (AA) metabolism. The effects of cholera toxin (CT) or pertussis toxin (PT), and nonhydrolyzable GTP analogs on *S. enteritidis* LPS stimulation of $iTXB_2$ and i6-keto-$PGF_{1\alpha}$ synthesis in normal resident peritoneal MØ and MØ from LPS tolerant rat were determined. Pretreatment with PT, which irreversibly ADP ribosylates G_i, had no effect on basal macrophage $iTXB_2$ or i6-keto-$PGF_{1\alpha}$ production, but PT significantly inhibited LPS-stimulated $iTXB_2$ or i6-keto-$PGF_{1\alpha}$ synthesis. Pretreatment with CT, which irreversibly ADP ribosylates G_s, significantly enhanced LPS-induced synthesis of TXB_2 or i6-keto-$PGF_{1\alpha}$ at doses of CT alone which did not affect basal synthesis. The stable GTP analog GTP-[γ-S] (100 μM) also significantly increased $iTXB_2$ synthesis and significantly augmented LPS-stimulated $iTXB_2$ synthesis. The effects of PT, CT or GTP-[γ-S] with or without LPS were markedly ($P<0.05$) depressed in MØ from LPS tolerant rats compared to control MØ. To further test the hypothesis that G protein function is altered, GTPase was measured in MØ from LPS tolerant rats. In LPS tolerant MØ, basal membrane GTPase measured by the liberation ^{32}P from (γ-^{32}P) GTP was reduced to one-fourth the values seen in control membrane basal cell levels. These composite results demonstrate that (1) G protein activation is necessary for LPS induction of AA metabolites in resident MØ and, (2) reduced G protein function may play a role in the molecular mechanism of desensitization to LPS. (Supported by NIH GM 27673).

CB 303 ROLE FOR PROTEIN KINASE C ACTIVATION IN THE DIRECT EFFECTS OF ENDOTOXIN AND PHORBOL ESTER ON VASOCONSTRICTION IN THE ISOLATED PERFUSED RAT LIVER, James P. Filkins, Julia Hunt and Joong-Woo Lee, Department of Physiology and The Shock-Trauma Institute, Loyola University Chicago Medical Center, Maywood, IL 60153

We recently reported (1) an augmentation of endotoxic lethality and glucose dyshomeostasis by phorbol esters in vivo, and (2) antagonism of endotoxicity by protein kinase C (PKC)inhibitors (Am J Physiol 260:R494-502 and 261:R26-31, 1991). The current study evaluated the direct vascular effects of phorbol ester vs endotoxin preparations on hepatic vasoconstriction in the isolated, portal perfused rat liver. The perfusate employed was Krebs-Ringer bicarbonate buffer gassed with 95% oxygen and 5% carbon dioxide. Phorbol 12-myristate 13-acetate(PMA), a potent activator of PKC, at concentrations of 2 to 200nM increased hepatic resistance. The resistance alterations were transient and reversible within 30 minutes. A second dose of PMA failed to alter hepatic resistance-i.e., tachyphylaxis occurred. Pre-treatment with the PKC inhibitors H-7 and polymyxin B blunted the PMA-induced vasoconstriction. Salm. enteritidis lipopolysaccharide B failed to alter hepatic resistance at concentrations up to 100 mg per liver; Salm. minnesota Re595 glycolipid in either a bolus (5mg) or infusion model (5mg over 10 min) increased hepatic resistance. A second dose of either the glycolipid or PMA failed to alter hepatic resistance-i.e., cross-tachyphylaxis occurred. In addition, the glycolipid induced vasoconstriction was also blunted by H-7 and polymyxin B. Therefore, the signal transduction system mediating endotoxic-induced vasoconstriction in the perfused liver probably involves activation of PKC. Thus one mode of protection of PKC inhibitors in endotoxic shock may entail prevention of hepato-splanchnic congestion. Since the liver is a major site of endotoxic recognition and processing, further analysis of hepatic cellular interactions offers promise of elucidating the fundamental action of endotoxin in an intact organ system which maintains critical cell to cell and cell to matrix integrity. (Supported by HL31163)

CB 304 HAEMOPHILUS INFLUENZAE LIPOPOLYSACCHARIDE DISRUPTS AN IN VITRO BOVINE BLOOD BRAIN BARRIER VIA A CYTOTOXIC PATHWAY INVOLVING SOLUBLE CD14, E. A. Frey[1], S. D.Wright[4], D. Patrick[1,2], J. Betts[1], R. Prameya[3], K. Dorovini-Zis[3], and B. B. Finlay[1], [1]Biotechnology Laboratory, Departments of Biochemistry and Microbiology and the C. B. D. N. Centre of Excellence, [2]Division of Infectious Diseases, [3]Department of Pathology, University of British Columbia, Vancouver, B.C. Canada. [4]Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York.

Purified bovine brain microvascular endothelial cells were used as an in vitro blood brain barrier (BBB) model to study the effect of Haemophilus influenzae type b (Hib) on the BBB. Cytopathic effects were seen with whole bacteria, heat-killed bacteria, bacterial filtrates, and lipopolysaccharide (LPS). Morphological changes in the monolayers included the rounding and detaching of cells, and the disruption of tight junctions. The same effect was seen with purified Escherichia coli LPS. In all cases the cytotoxic effect could be totally blocked with polymixin B. A number of cultured cell lines were tested, and similar cytotoxic effects were observed with the bovine pulmonary vascular endothelial cell line (CPAE). Visible cytotoxicity in both endothelial cell types was confirmed with a tetrazolium-based colorimetric assay (MTT). The presence of serum was found to be essential for the LPS-mediated cytotoxic effect. Similar cytotoxicity results were seen with human, monkey, horse, rabbit, bovine and fetal calf serum. A monoclonal antibody against the myeloid antigen CD14 completely blocked the cytotoxic effect of Hib LPS . CD14, a phosphatidylinositol (PI) linked receptor involved in mediating the effect of LPS-lipopolysaccharide binding protein (LBP) complex in monocytes, is also found in a soluble form in serum. Our results suggest that LPS and soluble CD14 have direct cytotoxic effects on bovine endothelial cells without the involvement of monocytic cells.

CB 305 UPTAKE OF LPS AND ENZYMATICALLY DEACYLATED LPS MEDIATED BY LPS BINDING PROTEIN AND CD14: INHIBITION OF LPS-INDUCED NF-kB ACTIVITY WITHOUT INHIBITION OF LPS UPTAKE.

Richard L. Kitchens and Robert S. Munford, Departments of Internal Medicine and Microbiology, University of Texas Southwestern Medical Center, Dallas, TX 75235-8859.

Deacylated LPS (dLPS) is produced by the action of the leukocyte enzyme, acyloxyacyl hydrolase, which removes secondary fatty acyl chains from the lipid A moiety of LPS. dLPS and its structural counterpart, lipid IVA, are non-toxic LPS antagonists in human cells, yet their inhibitory mechanism has not been determined. We used the human monocytic cell line, THP-1, to explore CD14-mediated uptake and response to physiologic concentrations (less than 10 ng/ml) of LPS and dLPS. ^{3}H-LPS or ^{3}H-dLPS was complexed to purified LPS binding protein (LBP) and added to THP-1 cells that expressed high levels of CD14 following 96 hr incubation with dihydroxy vitamin D_3. Uptake of the radiolabels by adherent cells was measured and induction of nuclear NF-kB binding activity was determined by gel-shift assay. Pre-bound dLPS:LBP complexes were taken up at least as efficiently as LPS:LBP in dose response and time course experiments, but 10 ng/ml dLPS:LBP did not induce NF-kB. In contrast, less than 0.1 ng/ml LPS:LBP produced an NF-kB response. In both cases uptake was inhibited by a monoclonal antibody to CD14. Greater than 50% inhibition of NF-kB was seen using equal amounts of prebound dLPS:LBP and LPS:LBP added simultaneously or sequentially (dLPS followed by LPS), yet LPS uptake was not decreased. Similar results were seen using synthetic lipid IVA instead of dLPS. TNF-induced NF-kB activity was not inhibited by dLPS. Surprisingly, the mechanism by which dLPS and lipid IVA inhibit the cellular response to LPS is not competition for LPS uptake via CD14. We propose that inhibition occurs by competition for another LPS-binding molecule (the signal-transducing LPS receptor ?), by competition at a subsequent step in the signal pathway, or by induction of an inhibitory signal.

CB 306 TNF GENE EXPRESSION IS GOVERNED BY THE UNTRANSLATED REGIONS IN NON-MACROPHAGE CELL LINES; IDENTIFICATION OF A TRANS-DOMINANT ACTIVATOR, Veronique Kruys, Kathleen Kemmer, Alexander Shakhov, Victor Jongeneel and Bruce Beutler, Howard Hughes Medical Institute,Dallas TX 752359050, Ludwig Institute for Cancer Research, Epalinges, Switzerland.

While tumor necrosis factor (TNF) is predominantly produced by macrophages, it is also expressed by non-macrophages, and even by extrahematopoietic cells. Such cells may constitute an important source of TNF, as this protein is produced in neoplastic disease. In order to study TNF gene regulation, we designed reporter constructs in which a CAT coding sequence was associated with different combinations of the mouse TNF promoter, 5' untranslated region (UTR), and 3' UTR. The role of these sequences in TNF gene expression have been examined in three non-macrophage cell lines (HeLa, NIH3T3 and L-929). The TNF promoter is not macrophage-specific since it is well utilized by each cell type. Strikingly, the TNF 3' UTR effectively cancels reporter gene expression in HeLa cells and NIH3T3 but fails to block expression in L-929 cells. L-929 cells contain a factor which overcomes the inhibitory effect of the 3' UTR. Its action depends upon the presence of the 5' UTR. Cell fusion experiments reveal that this factor is trans-dominant. These studies show the essential role played by the TNF 3' UTR in the suppression of TNF gene expression in cells that might otherwise express TNF. They also reveal the existence of an escape pathway whereby TNF might be inappropiately produced.

Data concerning the mechanism of expression in L-929 cells will be presented.

CB 307 VASCULAR SMOOTH MUSCLE CELL SURFACE-INTERLEUKIN 1 MAY REGULATE CYTOKINE PRODUCTION IN THE VESSEL WALL IN AN AUTOCRINE OR PARACRINE FASHION DURING INFECTION OR INFLAMMATION.

Harald Loppnow, Ernst Th. Rietschel, Helmut Brade, Hans-Dieter Flad, and Peter Libby*. Forschungsinstitut Borstel, D-2061 Borstel, Germany, and *Brigham and Women's Hospital, Boston, MA 02115, USA.

Vascular cells, such as endothelial (EC) and smooth muscle cells (SMC), might be involved in the regulation of host responses to endotoxin (LPS) by production of cytokines. EC and SMC produce interleukins (IL) like IL1 or IL6 (1, 2). During infection with Gram-negative bacteria LPS or LPS-induced macrophage-derived cytokines may stimulate vascular cells to produce mediators, which can in turn induce other cytokines. We show here, that in response to LPS, partial structures therof or cytokines SMC produce IL6, which is released almost quantitatively. LPS- or cytokine-stimulated SMC also produce IL1. In contrast to earlier observations these cells do not release IL1 into the culture supernatant, but retain this activity in the cytosol. It is also present on the cell surface of SMC as detected in membrane IL1-assay with D10-cells, according to the method used for fixed monocytes (3). The cell-surface IL1 of fixed SMC stimulated IL6-production as demonstrated by the addition of fresh SMC to fixed SMC. Fixed stimulated SMC induced higher amounts of IL6 than fixed unstimulated SMC. Addition of IL1 antibodies inhibited both the D10- and the SMC-response. These results indicate that during infection or inflammation surface-IL1, produced by SMC in the vessel wall, may be involved in the regulation of the complex cytokine cascade in an autocrine or paracrine fashion.

References: **(1) Libby et al., 1986, JCI, 78, 1432.**
(2) Loppnow and Libby, 1990, JCI, 85, 731.
(3) Kurt-Jones et al., PNAS, 82, 1204.

CB 308 SERUM DEPENDENCE OF PHAGOCYTE RESPONSES TO ENDOTOXIN W. A. Lynn and D.T. Golenbock. Division of Infectious Diseases, Boston City Hospital., Boston, MA 02118. Gram-negative bacterial septicemia is a common clinical syndrome resulting, in part, from the activation of phagocytic leukocytes by lipopolysaccharide. We have used flow cytometry to measure up-regulation of immunofluorescent CR3 (CD11b/CD18) on neutrophils and enzyme linked immunoassay to assay monocyte TNFα release as measures of granulocyte activation by LPS. After exposure to *Salmonella minnesota* R595 LPS, expression of immunofluorescent CR3 on the PMN surface rapidly increases, achieving a peak (typically 2-3 fold over baseline) by 30 minutes. The increase in CR3 expression was similar in kinetics and magnitude to that produced by FMLP, PMA and $rTNF_{tt}^{hu}$. We have established that the lipid A-like molecules designated lipid IV_A, KDO_2IV_A and deacylated LPS as well as lipid A from *Rhodobacter sphaeroides* are specific antagonists of ReLPS, both with PMN (*J. Immunol.*, in press) and monocytes (assessed by the release of cytokines, PGE2; *J. Biol. Chem* ., in press) thereby suggesting specific lipid A/receptor interactions. PMN and monocytes which were exposed to the LPS antagonists and then washed, were refractory to stimulation by LPS suggesting that the site of action of the inhibitors was the phagocyte outer membrane. The activation of phagocytes was dependent on the presence of serum: cells washed in RPMI/human albumin were 30-100 fold less responsive to LPS. However, PMN isolated in serum-free conditions could still be stimulated by higher concentrations of LPS. Monoclonal antibody directed against CD14 (mAb 3C10), specifically inhibited LPS induced CR3 expression on the surface of washed PMN *both in the presence and absence of autologous serum*. Because the ability of 3C10 to inhibit the response of washed PMN to LPS may be due to adsorbed (or receptor bound) serum proteins remaining on the surface of PMN, we have recently established a clone of the human myelomonocytic cell line THP-1 in a defined serum-free medium (SFM-1A). Initial studies with SFM-1A suggest that in the absence of serum, the cells are refractory to LPS at concentrations (10-100 ng/ml) which usually produce maximal cell stimulation, an effect which could be reversed by the addition of 1% serum. Higher concentrations of LPS from 10 -100 μg/ml were still able to stimulate SFM-1A despite the absence of serum. These findings support the hypothesis that LPS stimulation of phagocytes is enhanced by serum proteins and in the presence of serum involves CD14. However, serum-independent mechanisms of cell signalling by LPS also appear to be present.

CB 309 BLOOD NEUTROPHILS OF HUMANS WITH SEPTIC SHOCK ARE TOLERANT TO ENDOTOXIN-INDUCED EXPRESSION OF THE INTERLEUKIN-1 β GENE. Charles E. McCall, R. Guzman, L. Grosso-Wilmoth and S. Cousart. Wake Forest University Medical Center, Winston-Salem, NC 27157-1042.

Expression of the genes coding for interleukin-1 (IL-1) appears to be an essential pathogenic component of septic shock. IL-1 α and β genes are up-regulated in normal human blood neutrophils (PMN) stimulated by endotoxin (Lord, P. et al., J. Clin. Invest., 1991, 87:1312-1321); thus blood PMN could be an important source of IL-1 in septic shock. We report here that human blood PMN obtained from patients with septic shock are consistently tolerant to endotoxin-induced expression of the IL-1 β gene. Sepsis-PMN from all 21 patients with septic shock studied had marked reductions (50-100% of control) in synthesis of immunoreactive IL-1 β in response to 1-10,000 ng/ml 0111:B4 *E. coli* endotoxin ($p < 0.001$). In contrast, there was a consistent elevation in the number of low molecular weight Type II interleukin-1 receptors (IL-1 R2) on these sepsis PMN (7- to 100-fold above control) ($p<0.001$). Endotoxin tolerance of sepsis-PMN was stimulus-specific, since sepsis-PMN often normally synthesized IL-1 β in response to *S. aureus*. Reduced synthesis of IL-1 β in endotoxin tolerant sepsis-PMN correlated with decreased levels of IL-1 β mRNA in endotoxin-stimulated sepsis-PMN. Peripheral blood mononuclear cells from patients with septic shock were not tolerant to endotoxin-induced synthesis of IL-1 β. We speculate that the genes regulating expression of IL-1 R2 and IL-1 β may be under counter-regulatory controls that alter the PMN phenotype during septic shock; this might be an attempt by the host to protect itself against the adverse effects of endotoxin.

CB 310 EFFECTS OF INHIBITORS OF CYCLOOXYGENASE, LIPOXYGENASE AND PLA_2 ON ENDOTOXIN-PRIMED SUPEROXIDE RELEASE BY SEQUESTERED NEUTROPHILS AND KUPFFER CELLS. J.A. Spitzer and A.M.S. Mayer, Dept. of Physiology, LSU Med. Ctr., New Orleans, LA 70112. We have previously demonstrated that *E. coli* endotoxin (ET) infusion into rats for 1.5 and 3 hours primes sequestered neutrophils and Kupffer (K) cells for *in vitro* superoxide (SO) release in response to PMA stimulation, and causes significant shifts in their eicosanoid profile including both the cyclooxygenase (COX) and lipoxygenase (LOX) pathways (Infection and Immunity, Dec. 1991, J. Leuk. Biol. 48,488, 1990 and J. Leuk. Biol. S-2,30, 1991). To explore the possible mediating role of LOX and COX metabolites of arachidonic acid (AA) and AA as such, Indomethacin (Indo), (a COX inhibitor), ETYA, WY-50,295 and VZ-65 (LOX inhibitors), and manoalide (MNL) a powerful PLA_2 inhibitor were evaluated for their ability to block the priming effect of ET on SO release. Male, Sprague-Dawley rats were infused for 1.5 h with a nonlethal dose (268 μg/kg) of ET, or sterile saline. The hepatic sequestered neutrophils and K cells were recovered in the 45 ml/min elutriated fraction. SO release upon PMA (10^{-6}M) and opsonized zymosan (312 μg/ml) stimulation was measured in the absence and presence of the various inhibitors. No suppression of priming for PMA stimulation was noted by INDO, modest inhibition by the LOX inhibitors, e.g. 30% by 1 um VZ65, 10 uM WY-50,295 and 10 uM ETYA, but significant inhibition by MNL (IC_{50}=0.3 uM). Likewise, OPZ-stimulated SO release was not affected by INDO, modestly inhibited by WY 50,295 (40% at 1 uM) and strongly inhibited by MNL in a dose dependent- manner (40% inhibition at 0.5 uM, and 80% at 1 uM). The results suggest that PLA_2 activity and the implied AA release, rather than the metabolism of AA are associated with the enhanced SO release by hepatic neutrophils and K cells of ET-infused rats. Supp. by ONR grant N00014-89-J-1916 and NIH grant GM 32654.

CB 311 THE ROLE OF PLATELET ACTIVATING FACTOR IN AN ENDOTOXIN-INDUCED RABBIT MODEL OF INFLAMMATORY MENINGITIS, Joel Unowsky, Carol L. Meschter, Kim McIntyre, Christina A. Malangone, Judith M. Halpern, Karen E. Kowal, Departments of Antibacterial Research, Immunopharmacology, and Investigative Toxicology, Hoffmann-LaRoche, Inc., Nutley, NJ 07110

Inflammatory meningitis is induced by bacterial components including endotoxin and teichoic acid. These components induce the formation of a variety of inflammatory mediators. The precise role of specific mediators is under investigation. Whether PAF (platelet activating factor) plays a major role in the pathophysiology of inflammatory meningitis is controversial. We examined the role of PAF in an endotoxin model of inflammatory meningitis in rabbits.

Intracisternal injection of 12.5 or 0.5 ug of endotoxin (Salmonella minnesota Re 595) induced a dose dependent suppurative meningitis in young adult New Zealand rabbits. The endotoxin-induced meningitis was characterized by neutrophilic infiltration, edema and congestion of the meninges and superficial neuropil with multiple fibrin thrombosis of the meningeal vessels. At both dose levels, there was a sharp rise in tumor necrosis factor (TNF) which began at 1 hour and returned to normal at 3.5 hours. This was followed by a sustained rise in interleukin 1 (IL1) levels beginning at 4.5 hours. Significant cerebrospinal fluid (CSF) leukocytosis occurred at both levels of endotoxin. At the 12.5 but not the 0.5 ug level, a four-fold increase in platelet activating factor (PAF) levels was observed at 1 hour and continued over 24 hours. In rabbits administered 1.0 or 0.1 mg PAF intracisternally, mild CSF leukocytosis occurred at both levels. Minimal to mild suppurative inflammation of the meninges and CSF leukocytosis was seen at both dose levels of PAF and was significantly less than that induced by endotoxin. There were no significant increases in CSF TNF or IL1 in PAF-treated rabbits. Significant changes in the CSF glucose and total protein levels were not seen. However, lactic dehydrogenase and creatine kinase levels increased temporally and significantly over 24 hours. PAF antagonists (PAFa) including Ro 24-4736 have been shown to prevent pathology in other endotoxin-induced and PAF-induced conditions in a variety of animal models. The prophylactic intracisternal injection of 4 or 40 ug of the PAFa Ro 24-4736 did not prevent any effects of intracisternally injected endotoxin.

CB 312 LPS INDUCES TYROSINE PHOSPHORYLATION AND ACTIVATION OF MAP KINASES IN MACROPHAGES, Steven L. Weinstein, Jasbinder S. Sanghera, Anthony L. DeFranco and Steven L. Pelech, Department of Physiology and Department of Microbiology and Immunology, University of California, San Francisco, CA 94143 and the Biomedical Research Center, University of British Columbia, Vancouver, BC V6T1Z3 Canada.

Bacterial lipopolysaccharide (LPS) is a potent activator of responses by macrophages including the secretion of IL-1, TNF and arachidonic acid metabolites and the induction of anti-microbial activity. How macrophages are activated by LPS is incompletely understood. Previously, we have reported [PNAS 88:4148(1991)] that LPS rapidly increases protein tyrosine phosphorylation in murine macrophages and this event appears to mediate some macrophage responses to LPS. To further investigate the role of protein tyrosine phosphorylation in LPS-stimulated macrophages, we tested whether several 40-45 kDa proteins whose tyrosine phosphorylation is increased in RAW 264.7 cells following LPS treatment correspond to MAP kinase isozymes. This family of serine, threonine protein kinases is activated by tyrosine phosphorylation and they are thought to participate in signal transduction reactions in many cell types. LPS induced a large increase in MAP kinase activity, as measured by the capacity of appropriate fractions from a Mono Q column to phosphorylate the standard substrate myelin basic protein (MBP). These fractions contained two species of 42 kDa and 44 kDa which were reactive with specific anti-MAP kinase antibodies. Tyrosine phosphorylation of these species was also increased by LPS treatment. Conversely, inhibition of LPS-induced tyrosine phosphorylation of p42 and p44 was accompanied by decreased MBP phosphorylation. Thus, a strong correlation was observed between the status of p42 and p44 tyrosine phosphorylation and the level of MAP kinase activity. Similar results were obtained with smooth and rough forms of LPS and synthetic lipid A suggesting that activation of MAP kinases by LPS is lipid A dependent. In addition, this response does not appear to involve the activation of protein kinase C even though PMA also induced p42/p44 tyrosine phosphorylation and MAP kinase activation. We found that a PKC inhibitor, Compound 3, had no effect on LPS-stimulated p42/p44 tyrosine phosphorylation or activation of MAP kinase, but completely blocked both PMA-induced responses. Thus, our results suggest that LPS induces the tyrosine phosphorylation of at least two MAP kinase isozymes and that this modification of these proteins appears to increase their enzymatic activity. Since MAP kinases are thought to be regulators of cellular activation, these kinases may be important targets for LPS action in macrophages.

Therapy

CB 400 THE ROLE OF ENDOTOXIN IN DTP VACCINE-INDUCED HEPATOTOXICITY, Sherry Ansher, Walter Thompson and William Habig, Division of Bacterial Products, Center for Biologics Evaluation and Research, FDA, Bethesda, MD 20892.

Administration of a single human dose of Diphtheria and Tetanus Toxoids and Pertussis Vaccine Adsorbed (DTP) to endotoxin responsive (R) and nonresponsive (NR) mice is associated with increased hexobarbital-induced sleep time and inhibition of hepatic microsomal drug metabolism. The mechanism for these effects has not been elucidated, nor have the components responsible been identified. To investigate the role of endotoxin (LPS) in vaccine-induced hepatotoxicity, we have examined the effects of administration of pertussis LPS and DTP vaccine in both R and NR mice. Hexobarbital-induced sleep times were elevated 1.5- to 1.7-fold above controls in both strains of mice 24 hours after a single injection of DTP vaccine. In contrast, following a single i.p. injection of 50 ug LPS, there was a 1.2-fold increase in sleep time in NR mice compared with a 2.5- to 3.0-fold increase in R mice. Similar effects were also measured on spectral cytochrome P-450 levels. DTP vaccine reduced levels 60% in R mice, but only 15% in NR mice. Serum cytokines were measured from 1 to 24 hours after administration of vaccine or endotoxin. Significant increases in TNF and IL-6 were observed in mice treated with DTP vaccine. Levels of TNF and IL-6 were about 3-fold higher in R mice compared with NR mice. However, TNF was undetectable in NR mice treated with LPS, while in R mice, it was elevated to 6.5 ng/ml serum. This was more than 6-fold higher than the DTP treatment. Similar differences were also seen with IL-6. Furthermore, when polymyxin was used to neutralize endotoxin in the vaccine or LPS, the effects of LPS were almost completely eliminated, while those of the vaccine were reduced only slightly. These data suggest that although endotoxin may contribute to the toxicity of DTP vaccine, it is not the only component responsible for the observed adverse effects.

CB 401 MONOCLONAL ANTIBODIES TO KDO BIND WELL TO MANY SEROTYPES OF *K. PNEUMONIAE* LPS, B.J. Appelmelk, N. v.d. Meer, W. Nimmich, J.J. Maaskant, A.M.J.J. Verweij-van Vught, L.G. Thijs, D.M. MacLaren, Departments of Medical Microbiology and Acute Intensive Care, Free University, Amsterdam, The Netherlands and Institute for Medical Microbiology, University Rostock, Germany.

Klebsiella pneumoniae (Kpn) is an important cause of Gram-negative sepsis, a clinical situation still associated with a high mortality. Recent studies have shown that the capsule of Kpn is often permeable to anti-LPS antibodies, and thus adjunct immunotherapy with anti-LPS Abs would be feasible. Of great use would be monoclonal antibodies (Mabs), binding to common elements of the LPS core, and hence with a high affinity for all serotypes of Kpn LPS. A panel of 19 IgG and IgM Mabs was prepared following vaccination of mice with rough mutant LPS (mostly *Salmonella minnesota* R595, i.e lipid A + Kdo). Both Mabs specific for a α-2.4 linked Kdo-Kdo disaccharide (clones 25 and 27, both IgG) and for terminal α-linked Kdo monosaccharide (clones 14, 20 and 35, all IgM) were found to react with Kpn LPS. The reactivity of clone 20 was investigated in more detail in the passive hemolysis assay (PH), in Elisa and in inhibition versions thereof. All currently recognized serotypes (O1,O3-5,O7-10 and O12), represented by LPS purified from 17 reference strains, were included in the study. In PH, clone 20 reacted as well with several Kpn LPS as with R595 LPS (titers 4-16 ng/ml); two LPS did not react at 4000 ng/ml Mab. In Elisa, the same pattern was observed but here 5 out of 17 LPS did not react at 4000 ng/ml. Likewise, in inhibition of PH a range of affinities was observed. IN SUMMARY we have demonstrated that Mabs to Kdo bind to many serotypes of smooth Kpn LPS, and immunotherapy of Klebsiella infection might be feasible. The ability of these Kdo Mabs to block Kpn LPS-host interactions is under investigation.

CB 402 REACTIVITY OF CD5+ (B1) ANTIBODIES WITH RC LPS AND PEPTIDOGLYCAN; Neelima M. Bhat, Marcia M. Bieber and Nelson N. H. Teng, Department of Gynecology and Obstetrics, Stanford University School of Medicine, Stanford, CA 94305

The reactivity of a panel of CD5+ (B1) and CD5- human monoclonal antibodies (HMAb) to RcLPS, ssDNA and high molecular weight peptidoglycan (hMW PG) was studied. Human antibody was obtained from hybridomas and limiting dilution of EBV transformed B cells. B1 and conventional B cells were obtained from fluorescence activated cell sorter (FACS) sorted adult spleens. B1 cells were also obtained from human fetal spleens from 18-22 wks gestation. No conventional B cell MAbs or Abs from limiting dilution EBV transformed cells reacted with any of the above antigens (210 clones assayed). Approximately 5-8% of B1 cells produced Ab that reacted with RcLPS and ssDNA (250 clones assayed). All Abs that reacted with RcLPS reacted with ssDNA. Relative binding affinity was determined in solid phase Elisa. Of 20 B1 MAbs (all IgMs) with various affinities for ssDNA and RcLPS, 5 did not react with hMW PG. Of 20 B1 MAbs that did not react with ssDNA or Rc, none reacted with hMW PG. All fetal B1 MAbs that bound to Rc, ssDNA and hMW PG contained heavy chain variable region 3. The three fetal MAb that did not react with hMW PG were also VH3. Adult B1 MAbs were either VH3, VH4 or in one case VH1. These B1 antibodies may represent a form of primitive and broadly reactive immune response to bacterial infection.

CB 403 THE HUMAN MONOCLONAL ANTIBODY (MAb) HA-1A BINDS TO ENDOTOXIN VIA AN EPITOPE IN THE LIPID A DOMAIN OF LIPOPOLYSACCHARIDE (LPS), W.C. Bogard, Jr., P. Daddona E.M. Damiano, A.O. Leone, P. Kaplan, and S. Siegel, Centocor, Inc., Malvern, PA, 19355.

HA-1A, a human MAb (IgM,κ) has been shown to significantly reduce mortality in septic patients with gram-negative bacteremia including those with septic shock. To confirm the reported specificity of this MAb for the lipid A domain of LPS, several assay systems were developed including an enzyme-linked immunoadsorbant assay (ELISA), which measured the binding of HA-1A to lipid A adsorbed to a solid phase; a rate nephelometry assay, which measured the ability of HA-1A to bind and aggregate lipid A in solution, and a dot blot immunoassay, which measured the ability of HA-1A to interact with lipid A adsorbed to nitrocellulose. In all three assay systems, HA-1A bound to monophosphoryl lipid A prepared from S. minnesota R595 LPS in a dose-dependent manner. A panel of negative control human IgM monoclonal or polyclonal antibodies did not exhibit binding to lipid A in these assay systems. Several experimental approaches were also employed to demonstrate that the binding of HA-1A to lipid A in these assay systems was a well-behaved interaction between the antibody and lipid A. Both polymyxin B and murine IgG MAb (8A1) with a specificity for lipid A were able to competitively inhibit HA-1A reactivity with lipid A in a dose-dependent manner. Further, an anti-idiotype MAb developed against HA-1A was also able to competitively inhibit the binding of HA-1A to lipid A in these assay formats. These latter results conclusively establish that the binding of HA-1A to lipid A was mediated through the variable region. Comparable HA-1A reactivity against synthetic lipid A confirms that the HA-1A binding observed to the natural lipid A was not the result of protein or DNA contaminants in the latter. Finally, the reactivity of HA-1A against a variety of different biochemically related substances was tested. No significant reactivity was found. Collectively, these results verify that HA-1A binds specifically to the lipid A region of LPS by an interaction with the variable region of the MAb.

CB 404 WIDELY CROSSREACTIVE ANTI-LPS CORE MONOCLONAL ANTIBODIES HAVE LPS NEUTRALIZING PROPERTIES, Franco E. Di Padova, Robin Barclay* and Ekke Liehl#, Ernst Rietschel~ Preclinical Research, Sandoz Pharma, CH 4002 Basel, Switzerland, *SNBTS, EH3 9HB Edinburgh, Scotland, #Sandoz Forschungsinstitut, A1235 Vienna, Austria, ~Forschungsinstitut Borstel, D-2061, Borstel, Germany.

High affinity and widely crossreactive anti-E. coli core MAbs have been generated. These MAbs recognize as their minimal epitope, the Rc core of E.coli J5 LPS. In DOC-PAGE and blotting of smooth (S) and rough (R) LPS, these MAbs recognize both the ladder and the core structure. Therefore they recognize a public and common epitope in the LPS molecule. This epitope is also exposed in heat killed bacteria. One of these MAbs recognizes all E. coli clinical isolates from blood and isolates from urine and feces. In this study we show that this MAb is crossprotective in vitro and in vivo. At 100nM, this MAb is able to block lymphokine secretion (IL-6 and TNF) by mouse peritoneal cells stimulated with different S- and R-LPS (10pM). This MAb inhibits S- and R LPS induced fever in rabbits (dose 0.3-3 mg/Kg; time -30 min) and LPS induced mortality in galactosamine sensitized mice (10 mg/kg; time -2h). LPS contamination was not detected in MAb preparations as assessed by a sensitive LAL assay and pyrogenicity in rabbits. Crossprotection in vivo against several smooth and rough LPS was observed. This new class of widely crossreactive and cross-protective anti-core LPS MAbs demonstrates the existance of common and public epitopes in the core structures of Enterobacteriaceae.

CB 405 KINETIC-QCL AND VALIDATION OF BLOOD COLLECTION TUBES FOR ENDOTOXEMIA DETECTION. Maribeth A. Donovan and Ronald N. Berzofsky, BioWhittaker, Inc., Research and Development, Walkersville, MD 21793

Successful treatment of endotoxemia is dependent on early intervention. The 2-4 day period necessary to detect positive blood cultures is, in some cases, too long and can only imply endotoxemia. A rapid and specific detection method is necessary to ensure timely and proper diagnosis. The observation by Levin and Bang that endotoxin triggers the gelation of lysate from the amebocytes (blood cells) of the horseshoe crab, Limulus polyphemus, has led to the production of sensitive assays for endotoxin detection. Limulus Amebocyte Lysate (LAL) testing has advanced from the qualitative gel-clot assays to the quantitative chromogenic and kinetic assays. BioWhittaker kinetic-quantitative chromogenic lysate, K-QCL, is the latest development in LAL technology.

Endotoxin-free blood collection tubes are a necessity for the detection of endotoxin in plasma. Lot to lot differences in the endotoxin content of heparin-containing blood collection tubes have been observed using K-QCL. The endotoxin contents of five lots of 5 ml draw tubes ranged from <0.005 to 2.0 EU/ml. Appropriate diluants must be chosen to overcome inhibition of LAL by anticoagulants. In this study, 15 mM $MgCl_2$ was shown to be satisfactory.

K-QCL can be used in the validation of blood collection tubes. With a sensitivity of 0.005 EU/ml and an assay time of 80 minutes, K-QCL is an ideal candidate for the clinical detection of endotoxemia. Endotoxin-free blood collection tubes and a rapid test for plasma endotoxin will lead ultimately to improved prognosis for endotoxemic patients.

CB 406 SPECIFIC, HIGH AFFINITY BINDING OF LPS BY HUMAN BACTERICIDAL/PERMEABILITY INCREASING PROTEIN (BPI_{55}) AND THE RECOMBINANTLY DERIVED AMINO TERMINAL FRAGMENT (BPI_{23}), H. Gazzano-Santoro, P. Conlon, L. Grinna, A. Horwitz, B. Parent, T. Parsons, G. Theofan and P. Trown, XOMA Corporation, Berkeley, CA 94710. P. Elsbach and J. Weiss, New York University School of Medicine, New York 10016.

Among the many antimicrobial agents elaborated by polymorphonuclear neutrophils (PMN) is a 55 kD cationic protein known as bactericidal/permeability-increasing protein (BPI_{55}). This protein isolated from human PMNs has been shown to have potent bactericidal activity against a broad range of Gram-negative organisms and to bind LPS. An N-terminal fragment generated by limited proteolysis has been shown to have the bactericidal and anti-endotoxin activities of intact BPI_{55}. To further define the functional domains of BPI, a recombinant protein (BPI_{23}) which corresponds to the N-terminal sequence of BPI_{55} was produced and purified to homogeneity. BPI_{23} and BPI_{55} were found to have equivalent bactericidal activity against Gram-negative bacteria. Using *in vitro* binding assays, both BPI_{23} and BPI_{55} were found to bind to natural and synthetic lipid A, to LPS isolated from a series of rough *Salmonella* chemotypes and to LPS isolated from a broad range of wild-type, smooth organisms, including many clinical isolates. The binding of BPI_{23} to those toxophoric substances is specific, saturable and of high affinity with an apparent K_d of 3-5 nM for all ligands tested. Smooth form LPS is heterogeneous in terms of the number of O-antigen repeats. Using SDS-PAGE/Western analysis, BPI_{23} was shown to bind all molecular forms regardless of the length of the O-repeat. In addition, the two BPI forms had equivalent potency in inhibiting the actions of LPS in the *Limulus* amebocyte lysate assay (IC_{50} approximately 3-5 nM). These results confirm and extend our previous work demonstrating that the potent bactericidal activity and the endotoxin binding site reside in the amino terminal domain of BPI.

CB 407 ANTI-LIPID A MONOCLONAL ANTIBODY MEDIATED INHIBITION OF ENDOTOXIN-INDUCED CYTOKINE PRODUCTION *IN VITRO*, Carolyn C. Huntenburg, Mark A. Wisniewski, Inghwa S. Fang, J. Eric Bubbers, Baxter Hyland Division, Duarte, CA 91010.

Cytokines, especially Tumor Necrosis Factor (TNFα) and Interleukin-1 (IL-1ß), have been implicated as important mediators of the shock syndrome. In order to evaluate the therapeutic potential of the human anti-Lipid A IgM mAb SdJ5-1.17.15, a study was conducted to characterize the *in vitro* effects of the mAb on endotoxin-induced TNFα and IL-1ß production by hPBMC. Levels of TNFα and IL-1ß were measured in hPBMC culture lysates and the cytokine levels were quantitated using an enzyme linked immunoadsorbant assay (ELISA). LPS and lipid A were observed to induce maximum levels of cytokines at concentrations between 1-10ng/mL. Seventeen LPS and four lipid A types (13 smooth or wild type; 8 rough mutants) were screened. At concentrations of 3 μg/mL or greater, the mAb significantly inhibited induced TNFα and IL-1ß production by all LPS and lipid A types tested. Cytokine inhibition was reached when antibody:antigen equi-molar ratios or greater were achieved and was mAb dose dependent. Specificity of reaction was examined using a non-relevant human myeloma IgM. Although cytokine production was significantly lower in the presence of myeloma protein when compared to formulation buffer controls ($p < 0.05$), the anti-Lipid A mAb showed a greater inhibition of cytokine production than either control ($p < 0.05$; Neuman Keul's analysis). TNFα inhibition by the myeloma protein is suggested to be an Fc mediated phenomena since pre-incubation of hPBMC with purified Fc fragments resulted in a similar inhibitory profile as compared to the myeloma protein and was significantly less than that of the anti-Lipid A mAb. The anti-lipid A mAb inhibited TNFα production by LPS induced hPBMC when added simultaneously, suggesting the antigen:antibody reaction rate is greater than the LPS:receptor interaction rate. The ability of the anti-Lipid A human mAb to modulate cytokine production *in vitro* suggest that this mAb may be a beneficial therapeutic agent in the treatment of Gram negative septic shock.

CB 408 IN VITRO INHIBITION OF LIPOPOLYSACCHARIDE (LPS) INDUCED TNF α PRODUCTION BY HA-1A (CENTOXIN™), A HUMAN MONOCLONAL IGM ANTI-ENDOTOXIN ANTIBODY, Peter Katsikis*, Gail Harris*, Ewa Paleolog*, Scott Siegel[1], Margaret Dalesandro[1], Peter Daddona[1] and Marc Feldmann*. *Charing Cross Sunley Research Centre, London, U.K., [1]Immunobiology R&D, Centocor, Inc., Malvern, PA 19355.

The human IgM monoclonal anti-lipid A antibody HA-1A has been shown to provide protection against the deleterious effects of lipopolysaccharide (LPS) in animal models and clinical trials with patients with Gram negative bacteremia and septic shock. Utilizing monocyte enriched peripheral blood we could demonstrate an HA-1A dose responsive inhibition of E. coli J5 LPS induced immunoreactive TNFα whilst a control human IgM monoclonal (anti-CMV) antibody failed to show any such inhibition. The blood preparations employed in this system were enriched for white blood cells as a whole (x2-3) but even more so for monocytes (x5-10) using a Fenwal Plasmatec extractor. The inhibition was shown to be mediated via the antigen binding site of HA-1A since it could be completely abrogated by $F(ab')_2$ fragments of anti-HA-1A mouse monoclonal anti-idiotype 9B5.5. The maximum inhibition after 24 hours of stimulation at 1 μg/ml of LPS was approximately 70%. This inhibition could also be observed with LPS from antigenically distinct species other than the E. coli J5 mutant such as S. minnesota R595, E. coli 0111:B4 and Klebsiella pneumoniae LPS, suggesting that in this in vitro system, HA-1A is effective against LPS from different Gram negative bacteria which only have in common core lipid A. Studies to elucidate the mechanism by which HA-1A is acting to inhibit LPS induced TNFα in this system are underway.

CB 409 MOLECULAR CHARACTERIZATION OF ANTIGEN BINDING PROPERTIES OF ANTI-LIPID A IgM MONOCLONAL ANTIBODY, Mohammad Kazemi, Richard L. Jackson, Carolyn C. Huntenburg, and J. Eric Bubbers, Baxter Hyland Division, Duarte, CA.

The binding properties and specificity of the Hyland SdJ5-1.17.15 human monoclonal IgM antibody (mAb), prepared against *S. minnesota* R595 lipopolysaccharide (LPS) was assessed by "dot blot" assay *in vitro*. Preliminary dot blot results indicated that the mAb reacted only with the isolated lipid A component of LPS, but not with the LPS itself. The reaction was specific and dose-dependent in respect to both antigen and antibody concentrations. The negative LPS binding results on dot blot assay may be attributed to possible adverse effects of solid phase on epitope exposure. This hypothesis was tested by inhibition studies in which mAb was first, in liquid phase, adsorbed with saturating levels of the test antigen (e.g. lipid A, LPS types and heat killed bacteria), and subsequently used for solid phase lipid A reaction in the dot blot assay. Liquid phase adsorption abolished or greatly reduced the specific anti-Lipid A mAb recognition of solid phase lipid A. This phenomenon, in turn, suggested that the antibody binding site (epitope) within the structure of adsorbing antigens was being recognized by anti-Lipid A mAb. It appeared that antigen presentation greatly effected the epitope structure. In order to identify the epitope sites, the reactivities of anti-Lipid A mAb with various lipid A analogues of known structural differences were tested. Preliminary results indicated that a combination of the fatty acid side chains and phosphate groups of Lipid A (both groups being implicated in sepsis mediation) of lipid A were apparently involved in the epitope structure. The mAb examined in this study was found to be directed against a conserved epitope of lipid A expressed on all gram negative strains tested. The cross reactive properties of this mAb make it attractive for evaluating its therapeutic potential.

CB 410 HA-1A (CENTOXIN™) A HUMAN MONOCLONAL IGM ANTI-ENDOTOXIN MAB MEDIATES IMMUNE ADHERENCE OF E. COLI J5 LPS VIA CR1 OF HUMAN RBC'S AND NEUTROPHILS, Jeffrey I. Krieger*, Robert C. Fletcher*, Scott A. Siegel*, Douglas T. Fearon[1], Donald S. Neblock*, Raymond H. Boutin*, Ronald P. Taylor[2], and Peter E. Daddona*, *Immunobiology R&D, Centocor, Inc., Malvern, PA 19355, [1]Department of Molecular Biology & Genetics, Johns Hopkins University, School of Medicine, Baltimore, MD 21205 and [2]Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, VA 22908.

HA-1A (Centoxin™) has been shown clinically to decrease mortality in septic patients with Gram negative bacteremia. A possible mechanism of HA-1A action could be binding to Gram negative bacterial endotoxin and lowering its bioavailability via complement fixation and clearance mechanisms. In this study we have evaluated the ability of HA-1A to augment the immune adherence of ^{125}I E. coli J5 lipopolysaccharide (LPS) in the presence of human serum selected for low titer of endogenous anti-J5 activity (representing ~20% of normals) to human RBC's and neutrophils. In vitro studies indicate that: 1) HA-1A (0.1-10 μg/ml) mediates immune adherence of ^{125}I J5 LPS to human RBC's and neutrophils in a dose dependent and time dependent manner. Under these conditions utilizing human RBC's at 10^9/ml, high concentrations of LPS (600 ng/ml) could be specifically bound. 2) Immune adherence occurred via the classical complement pathway as demonstrated by its calcium dependence; HA-1A-J5 LPS-C' immune complexes (IC) were shown to bind to CR1 on RBC's and neutrophils. 3) Time and temperature-dependent human neutrophil binding and internalization of IC was demonstrated by trypsin stripping of externally bound IC. These studies support the proposal that clinically attainable levels of HA-1A can lower bioavailability of endotoxin by mediating binding and potential clearance of LPS via human RBC's through the RES or via direct internalization by peripheral blood neutrophils.

CB 411 MOLECULAR CHARACTERIZATION OF RABBIT CAP18– A UNIQUE LIPOPOLYSACCHARIDE BINDING PROTEIN.

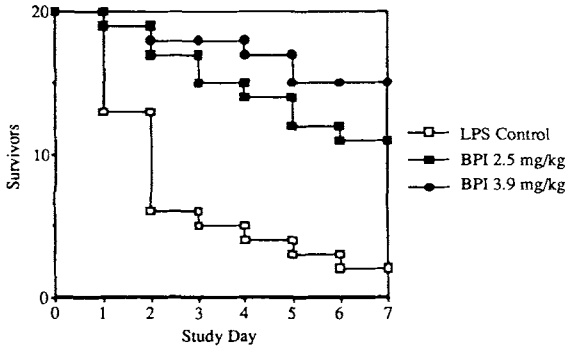
James W. Larrick[1,2], J.G. Morgan[2], I. Palings[2], M. Hirata[3], and M.H. Yen[2], S.C. Wright[1]; [1]Palo Alto Institute for Molecular Medicine, POB 8102, Stanford, CA 94309-8102. [2]Genelabs Inc., Redwood City, CA 94063; [3]Iwate Medical Univ., Morioka, Japan.

A rational approach to the control of Gram-negative sepsis is to neutralize the toxic effects of endotoxin or LPS. Cationic antibiotics such as polymyxin B bind to and neutralize some types of LPS, however clinical use is limited by toxicity. Monoclonal antibodies made to the cross-species conserved regions of LPS, such as the lipid A core, fail to neutralize smooth LPS, possibly due to steric effects by the LPS type-specific polysaccharides. Recently, several proteins have been identified that bind to and/or neutralize the toxic effects of LPS. These include mammalian BPI derived from granulocytes and LBP an acute phase protein produced primarily by the liver. In addition the LPS binding protein of the horseshoe crab, *Limulus* has been purified. CAP18 is a 18 kDa cationic protein originally purified by Hirata et al. from rabbit granulocytes using as an assay the agglutination of lipopolysaccharide (LPS) coated erythrocytes. CAP18 attenuates the activity of LPS in a number of assays. CAP18 binds to core mutant and smooth LPS coated onto erythrocytes. Purified CAP18 attenuates LPS induced generation of tissue factor in vivo and blocks lethality of LPS in galactosamine sensitized mice. cDNA clones encoding CAP18 were isolated from a rabbit bone marrow cDNA library using a PCR generated oligonucleotide probe derived from the N-terminal amino acid sequence[1]. The deduced amino acid sequence reveals a putative signal sequence of 29 amino acids and a mature protein of 142 amino acid residues. The predicted size of the encoded protein is 16.6 kDa with a pI of 10. There are no N-linked glycosylation sites. The CAP18 sequence bears no homology with other known LPS-binding proteins including human BPI and rabbit LBP. Comparison of the LPS binding domains of the various LPS binding proteins may lead to novel therapeutic strategies to neutralize LPS.

[1]Larrick JW et al. Biochem Biophys Res Comm 179:170-175, 1991.

CB 412 PREVENTION OF LETHAL ENDOTOXEMIA BY BPI_{23}, A RECOMBINANT 23 KD LPS-BINDING FRAGMENT OF BACTERICIDAL/PERMEABILITY INCREASING PROTEIN (BPI), W. Leach, V. Mohler, L. Grinna, A. Horwitz, G. Theofan, C. Mallari and A.H.C. Kung, XOMA Corporation, Berkeley, CA 94710.

LPS-binding and neutralization properties, as well as the bactericidal activity of the human neutrophil protein, BPI, have been shown to reside in a 25 kD N-terminal fragment. Accordingly, the corresponding gene was cloned and engineered for expression in mammalian cells. The resulting protein, BPI_{23}, which retains the *in vitro* biological properties of the natural 25 kD fragment, was tested for its ability to protect actinomycin D-sensitized CD-1 mice against lethal challenge with *E. coli* O111:B4 LPS. Each animal (20 mice/treatment group) received an intravenous injection of a mixture of actinomycin-D (0.8mg/kg) and LPS (1.0 μg/kg) followed by buffer (control), or BPI_{23} at doses of 2.5 and 3.9 mg/kg.



Mice treated with BPI_{23} were significantly protected ($p<0.005$) from the otherwise lethal endotoxin challenge. These results provide additional evidence that recombinant BPI_{23} may be a potent therapeutic agent for the treatment of endotoxemia and septic shock.

CB 413 A TNF RECEPTOR-IgG HEAVY CHAIN CHIMERIC PROTEIN AS A BIVALENT, LONG LIVED ANTAGONIST OF TNF ACTIVITY.

Karsten Peppel, David Crawford and Bruce Beutler. Howard Hughes Medical Institute, UT Southwestern Medical Center at Dallas. 5323 Harry Hines Blvd, Dallas TX 75235.

Using PCR, we linked the extracellular domain of the human 55 kD TNF receptor to the Fc portion (hinge, CH2-CH3) of a murine IgG1 heavy chain. Interposed between the two domains is a thrombin sensitive peptide linker. This contruct was expressed in CHO cells under control of the CMV promoter. The secreted protein is dimeric and capable of binding and inactivating TNF of human and murine origin. The molecule could be cleaved with thrombin to yield the TNF receptor extracellular domain in a pure form. However the extracellular domain has a greatly reduced TNF inhibitory activity as compared to the dimeric molecule. The bivalent molecule is also far more effective than neutralizing monoclonal antibodies in preventing TNF induced cytotoxicity in vitro. While the extracellular domain alone has a very short half-life in vivo, the half-life of the dimeric molecule is comparable to that of a murine monoclonal antibody. This molecule may prove very useful as an antagonist to TNF in vivo and as a means to decipher the mechanisms of interaction between TNF and its receptor.

CB 414 RECOMBINANT ENDOTOXIN NEUTRALIZING PROTEIN FROM *L. POLYPHEMUS* REDUCES MORTALITY OF GRAM NEGATIVE SEPSIS IN RAT MODELS, G. Siber, D. Nelson, N. Kupperman, C. Thompson, B. Hammer, N. Wainwright, T. Novitsky, A. Onderdonk, G. Fleisher. Children's Hospital, Dana-Farber Cancer Institute and Channing Laboratories, Harvard Medical School, Boston, MA 02115 and Associates of Cape Cod, Falmouth, MA 02540.

We previously showed that native Limulus anti-LPS factor, an 11.8 kDa protein isolated from amebocytes, significantly improved hypotension, acidosis, and mortality after an LD90 dose of meningococcal LOS in rabbits, even when given 30 minutes after challenge. A recombinant version of this protein, termed endotoxin neutralizing protein (ENP) similarly protected rabbits from an LD80 dose of *E. coli* LPS. To mimic human clinical sepsis more closely, we evaluated the effect of ENP on mortality in models of live bacterial infection. In infant rats given $1x10^4$ *H.influenzae* type b ip on d5 of life, ENP significantly delayed mortality despite persistent bacteremia at $>10^5$ cfu/ml:

ENP dose (mg/kg):	0	3	15	75	300
Mortality on d1:	12/18† (67%)	6/11† (55%)	12/19† (63%)	7/21† (33%)	5/20*† (20%)
Mortality on d2:	18/18 (100%)	11/11 (100%)	18/19 (95%)	17/21 (81%)	18/20 (90%)

*p<.05 vs 0 ENP by Fisher's Exact test; †p<.005 by trend analysis.

In Wistar rats implanted ip with $2.5\text{-}7.5x10^7$ *E. coli* O18ac K1 in gelatin capsules containing sterile cecal contents as an adjuvant, mortality was 100% without treatment. Gentamicin alone (5mg/kg) given 1h after challenge significantly reduced mortality (p<.01) and addition of ENP given 30 or 60 minutes after challenge further reduced mortality:

		Gentamicin + ENP (mg/kg)			
	No Rx	0	5	25	50
Mortality on d1:	22/22 (100%)	17/24† (71%)	12/24† (50%)	10/23† (43%)	5/23*† (22%)

*p<.01 vs 0 ENP by Fisher's Exact Test; †p<.002 by trend analysis.

We conclude that ENP may act synergistically with antibiotics in reducing mortality of gram negative sepsis in rats. Direct comparisons between ENP and IgM monoclonal antibodies to lipid A in these models are planned.

CB 415 THE HUMAN ANTI-ENDOTOXIN MONOCLONAL ANTIBODY HA-1A (CENTOXIN™) BINDS TO ROUGH GRAM NEGATIVE (GN) BACTERIA AND TO SMOOTH GN LABORATORY STRAINS AND CLINICAL ISOLATES AFTER ANTIBIOTIC BACTERIOLYSIS, Scott A. Siegel*, Martin E. Evans[1], Matthew Pollack[1], Ann O. Leone*, Cheryl Ann Kinney*, Susan H. Tam*, and Peter E. Daddona*, *Centocor, Inc., Malvern, PA 19355, and [1]F. Edward Hebert School of Medicine, Bethesda, MD 20814.

The human anti-endotoxin monoclonal antibody HA-1A reduces mortality associated with sepsis and GN bacteremia. HA-1A binds to the conserved lipid A domain of endotoxin, or lipopolysaccharide (LPS). In a liquid-phase immunoassay, HA-1A bound with high functional affinity to rough GN organisms such as E. coli D21F2 Re chemotype strain ($K_a = 3.1 \times 10^9 M^{-1}$). In contrast, HA-1A exhibited modest binding to smooth GN organisms. This binding was substantially increased when the smooth organisms were exposed to inhibitory concentrations of cell wall-active antibiotics. Binding to antibiotic-treated smooth GN bacteria was antibody and antibiotic dose-dependent. HA-1A did not bind to antibiotic-treated or untreated Gram positive organisms. Specificity of HA-1A binding to antibiotic-treated smooth GN organisms was documented by blocking experiments with lipid A, polymyxin B, and a mouse anti-idiotypic MAb that binds to the antigen-combining region of HA-1A. The enhancement of HA-1A binding was maximal at bactericidal concentrations of antibiotic. Flow cytometry demonstrated HA-1A binding to bacterial fragments under these conditions. Enhanced HA-1A binding was observed with phylogenetically diverse GN laboratory strains and 15 low-passage clinical isolates. Clinically, GN bacteria are exposed to antibiotics and other potentially lytic processes. These findings suggest that such interactions can enhance HA-1A binding to LPS from a wide variety of clinically relevant smooth GN bacteria, and are consistent with the observations concerning HA-1A benefits in clinical GN sepsis.

CB 416 LPS-INDUCED MACROPHAGE LEISHMANICIDAL EFFECT IN VITRO. D. M. Yang, A. Severn and F. Y. Liew, Department of Immunology, University of Glasgow, Western Infirmary, Glasgow, G11 6NT, U.K.

Murine macrophages activated with IFN-γ or TNFα in the presence of LPS produce high levels of nitric oxide and are strongly leishmanicidal. Neither IFN-γ or TNFα alone was effective in killing the intracellular parasite or inducing detectable levels of nitric oxide, suggesting that a second signal generated by LPS is required. Freshly isolated T cells and cloned T cell lines from susceptible BALB/c mice with progressive disease were stimulated with leishmanial antigens in vitro. These activated T cells significantly enhanced the growth of L. major in macrophages. This effect is reversible by the addition of LPS in the culture medium. Conversely, similarly activated T cells from the resistant CBA mice induced a strong leishmanicidal effect which was increased by LPS. These results suggest that additional effector mediators may play an important role in the host response to leishmanial infection. These mediators, which have LPS-like function, appear to be produced by the T cells from resistant mice but not by the susceptible strains of mice.

Late Abstract

SOLUBLE CD14 - A NEW THERAPEUTIC CONCEPT AGAINST SEPTIC SHOCK, Christine Schütt, Thomas Schilling, Christian Krüger, Felix Stelter and Uwe Grunwald, Department of Medical Immunology, Ernst-Moritz-Arndt-University, Greifswald, Germany

The relationship between expression of CD14 on cell surfaces (mCD14) and the amount of soluble CD14 (sCD14) present in the microenvironment, seems to be important fo cell activation induced by endotoxins. CD14 is a receptor for serum lipopolysaccharide binding protein/lipopolysaccharide (LBP/LPS) complexes (WRIGHT et al., Science, 249 (1990), 1431). Other LPS receptor exist too.

The mechanism by which mCD14 mediates cell activation by LPS in cooperation or competition to other receptors is not fully understood. Downregulation of mCD14 was realized by IFNY and IL4 in vitro. We observed in prefinal stages of septicaemia a total loss of mCD14 on patients monocytes (SCHÜTT et al., Mikroökol.Ther., in press). The mRNA expression and release of sCD14 in vitro was upregulated by LPS and TNFα, but down regulated by IL4. An initial decrease followed by an elevation of sCD14 serum levels we observed in follow up studies of polytraumatized and severely burned patients (KRÜGER et al., Clin exp. Immunol. 85, (1991), 297) suggesting a physiological counterregulatory mechanism.

Soluble CD14 is a functionally active receptor which binds opsonized LPS. It reduces dose dependently the LPS inducible generation of reactive oxygen species of human monocytes in vitro, even in the case of CD14 negative monocytes obtained from a patient suffering form PNH, which were highly responsible to endotoxin. This endotoxin neutralizing capacity of sCD14 seems to be independently from the receptor nature used by endotoxin on the cell surface.

Therefore sCD14 is a new therapeutic concept for prevention of septic shock.