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 Annual Report/Progress Summary
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I. Work Summary

Much has occurred over since our last progress report and over the past year. As outlined in our initial grant, we have continued to focus on proteins and peptides that bind endotoxin and which are therefore candidates for the treatment of septic shock. Our grant was planned to terminate on June 30, 1992. In part because of a slow startup time and in part because of careful cost management, we had excess funds in the grant and requested an extension of the grant until June, 1993. It is anticipated that we will exhaust our funds in the winter of 1993.

Our work over the past year has been divided between three main subjects:

- A. Analysis of monoclonal antibodies directed to lipid A
- B. Identification of the binding site of natural proteins that bind LPS through the use of overlapping synthetic peptides
- C. Study of the interaction of LPS with immunoglobulin in the presence of serum

We believe that we have made important progress in each area. A brief summary follows.

A. Analysis of monoclonal antibodies directed to lipid A

Although many investigators and companies have made monoclonal antibodies directed to LPS, two companies produced monoclonal antibodies directed to lipid A which are being actively developed for the treatment of septic shock. Centocor (Malvern, PA) is developing an human IgM antibody called HA-1A and Xoma (Berkeley, CA) is developing a murine IgM antibody called E5. HA-1A has been studied in a single clinical trial. Although it was initially reported to increase survival in patients with gram negative bacteremia, the methodology used in the trial is controversial. A second trial is underway studying the protective efficacy of HA-1A in patients with septic shock. E5 has been studied in two clinical trials. In the first trial, E5 was associated with a decrease in mortality in a small subset of patients who were not in refractory shock. In a second trial that focused on this group (sepsis without shock) E5 was not associated with decreased mortality.

Because each of these monoclonal antibodies is hypothesized to work by binding to LPS and neutralizing its action, we utilized the reagents and techniques developed in our grant to study the ability of each to bind and neutralize a wide variety of LPS from clinically relevant gram negative strains.

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As assessed by radioimmunoassay, both HA-1A and E5 bound tritiated LPS from multiple gram negative strains better than several control monoclonal and polyclonal antibodies. However the binding was very modest compared to polyclonal IgG directed to the O-polysaccharide of LPS. We studied the ability of HA-1A and E5 to neutralize LPS as assessed by the Limulus lysate assay, the mitogenicity of LPS for murine splenocytes, and the production of cytokines IL-1, IL-6, and TNF from human macrophages. We found that neither of the antibodies neutralized LPS in any of the assays, even in high concentrations. We therefore conclude that both antibodies bind LPS slightly, but do not neutralize its biological activity. These results have been written up and are accepted for publication in J. Exp. Medicine. A copy of the manuscript is included in the appendix.

B. Identification of the endotoxin binding site of several natural proteins that bind endotoxin

There are few natural proteins that bind LPS with high affinity. Three that have received intensive study over the last five years are Limulus anti-LPS factor (LALF, also called endotoxin neutralizing protein or ENP), LPS binding protein (LBP) and bacteriocidal permeability inducing protein (BPI). Each of these proteins alters the biological activity of LPS, and therefore is a candidate for therapeutic manipulation. However, the LPS binding site for each is unknown. To address this issue, we collaborated with Dr. Marek Kloczewiak to make overlapping synthetic peptides mimicking the amino acid sequence of each of these proteins. In this effort we were guided by charge and hydropathic plots of the proteins to help predict logical binding sites.

We utilized a capture Western blot system to measure binding of LPS to each of the synthetic peptides. This system has been described in detail in prior reports. Briefly, dilutions of the peptides were blotted onto nitrocellulose paper and then treated sequentially with LPS and rabbit anti-LPS IgG. Anti-LPS IgG was detected by goat anti-rabbit IgG and an avidin-biotin detection system. The lowest amount of peptide which captured LPS was measured. Using this system, we were able to identify the LPS binding domain of LALF. We are in process of mapping the exact binding site of LBP. These data are summarized below.

1. Limulus anti-LPS factor (LALF, ENP)

We have studied LALF extensively. We found that LALF binds LPS with high affinity and neutralizes its activity in multiple *in vitro* and *in vivo* assays. LALF protects against the toxic effects of LPS in mice, rabbits, and sheep. These results were published in June, 1992 in *Infection and Immunity*.

To identify the LPS binding site of LALF, we initially made four overlapping synthetic peptides mimicking the entire LALF protein. Binding activity was predominantly in the second peptide. We then mapped the binding domain by creating overlapping peptides around this peptide. Each binding assay was repeated three times. This information is portrayed in the appended table 1. Binding activity is represented as the lowest quantity of peptide that binds LPS in the assay. Activity is concentrated in a 14 amino acid sequence. This peptide does not neutralize LPS *in vitro*. However, as noted in our last report, a slightly larger peptide containing this sequence protects mice against LPS challenge, suggesting that the peptide may protect by clearing endotoxin from the bloodstream.

2. LPS binding protein (LBP) and bacteriocidal/permeability inducing protein (BPI)

These two LPS binding proteins are under intense investigation. BPI and LBP share greater than 50% sequence homology at the amino acid level. BPI is a cationic protein found in the granules of granulocytes. It is bacteriocidal for gram negative bacteria, and functions by binding to LPS in the wall of the bacteria causing permeability changes. It neutralizes LPS in numerous assays. LBP is an acute phase protein produced by hepatocytes that complexes with LPS in serum and then binds to the macrophage surface by CD14. In contrast to BPI, it increases the activity of LPS on macrophages (and probably other cells), and therefore increases cytokine production in response to LPS.

As above, to identify the LPS binding portion of these two homologous proteins, we reviewed charge and hydropathy plots for each. A clue to the binding site was that plasmin cleaved rabbit LBP between AA 99 and 100. We focused on the published human sequence of LBP and initially synthesized 5 peptides based on the first 102 amino acids of human LBP. We next studied the ability of the 5 slightly overlapping peptides to bind LPS using the blotting system described above. These data are portrayed in appended table 2. Two overlapping peptides (of 27 and 17 amino acids) have strong binding activity. In collaboration with Dr. Jean-Marc Cavallion, we also tested the ability of these same peptides to alter the cytokine production from human macrophages. In preliminary studies, the two peptides that bind also inhibit the secretion of LPS-induced tumor necrosis factor from the macrophages.

We are in the process of mapping the LPS binding site in more detail by synthesizing another series of peptides to pin down the exact site. Since LBP and BPI are homologous and bind LPS, we are hopeful that we are identifying the binding site of both proteins. Because the number of proteins that bind LPS with high affinity are limited, we were curious to see if there is any similarity of the binding sites between the LALF binding site and the binding site of LBP/BPI. Accordingly, we have started to study whether antiserum raised to one binding site reacts with the other. In two preliminary experiments, IgG raised to a synthetic peptide containing the active site of LALF cross-reacts with another synthetic peptide containing the internal sequence of the binding site of LPS. Controls included normal IgG and IgG directed to irrelevant bacterial antigens and an extensive panel of other synthetic peptides. While still preliminary, these experiments suggest that there is molecular mimicry between the LPS binding site in the Limulus protein and the two human proteins.

We are excited by all of these results. The identification of the LPS binding site of these proteins should help us to develop therapeutic agents based on blocking or clearing LPS.

C. Study of the interaction of LPS with immunoglobulin in the presence of serum

In the first years of the grant we found that tritiated LPS precipitates in polyclonal antisera raised to rough mutant bacterial vaccines, such as *E. coli* J5. We believe that this interaction is important to understand for two reasons. First, this antisera protects against LPS from all gram negative strains in animal models. However, there is no *in vitro* test that measures

binding. An understanding of the binding should lead to a means of developing monoclonal antibodies that may convincingly bind and that should protect. Second, knowledge of the interactions of LPS in serum should facilitate the development of any agent that is based on binding to the physicochemical forms of LPS that exist in serum. Indeed, the techniques that we have developed in the course of the grant have been extremely helpful in the analysis of several therapeutic agents that are being developed.

Over the last year we have made progress in understanding the binding that takes place in this antiserum. Approximately 60% of 5 ug/ml of tritiated LPS purified from 11 gram negative bacterial strains that we have studied precipitates in antiserum to E. coli J5, compared to 3-5% in normal serum. IgG purified from the antiserum reproduces the activity, but only in the presence of normal serum. Analysis by SDS PAGE of the precipitate formed in different conditions suggests that the IgG is a cryoglobulin. IgG remaining in solution in the cold has minimal activity, whereas IgG precipitated in the cold and resuspended at 37 degrees Centigrade has considerable activity. To study the relationship of time following immunization to activity, we immunized rabbits with E. coli J5 vaccine and prepared serum from the rabbits at different times after the primary vaccination. We found maximum precipitation for several weeks following the primary series, following which there was rapid loss of activity despite maintenance of IgG titers directed to E. coli J5 LPS by ELISA. Booster vaccinations again increased activity. These experiments confirm that ELISA titers directed to rough LPS do not correlate with binding activity. This ELISA was the assay used to screen for the currently available anti-LPS monoclonal antibodies. The fact that IgG, as opposed to IgM, has potent LPS binding activity and the timing of the antibody appearance should be very helpful in the development of monoclonal antibodies. These data are ready for publication.

II. New Knowledge since year #2

1. Two IgM monoclonal antibodies apparently directed to lipid A (HA-1A and E5) bind slightly to LPS as assessed by RIA, but do not neutralize its biological activity in a variety of biological assays.
2. We have mapped and identified the LPS binding sites for three proteins that bind LPS with high affinity using overlapping synthetic peptides and a sensitive blotting assay. These proteins are:
 - a. Limulus anti-LPS factor (LALF, ENP),
 - b. Bacteriocidal/permeability inducing protein (BPI), and
 - c. LPS binding protein (LBP).Fine mapping for the exact sequence of LBP and BPI are underway.
3. A peptide mimicking the active site of LALF binds LPS and protects in an actinomycin D sensitized mouse model. However, it does not neutralize LPS *in vitro*, suggesting that it may function by clearing LPS from the bloodstream.
4. This peptide has been chemically linked to human and murine IgG using bifunctional linkers in order to create peptide-immunoglobulin hybrid molecules. In preliminary studies, this molecule behaves like an LPS binding immunoglobulin as assessed by ELISA.
5. IgG directed to a peptide containing the LALF (ENP) binding site cross-reacts with a peptide containing the LBP binding site in two preliminary experiments. If confirmed, this finding suggests that the binding site has been highly con-

served over time (between *Limulus* crabs and humans).

6. In an attempt to understand the basis of protection of polyclonal antiserum to *E. coli* J5, we have been focussing on the interactions of ³H-LPS and elements in this antiserum. The development of monoclonal antibodies to the common antigens on the LPS core was based on the protective activity of this antiserum. We have established that:

a.. IgG from this antiserum binds to LPS. IgM from this antiserum also binds, although the interaction seems less.

b. The binding of IgG to LPS is critically dependent upon a factor in normal serum. The identity of this factor is unknown.

c. The IgG that binds to LPS is a cryoglobulin.

d. The fraction of IgG that binds LPS is induced rapidly after a primary immunization series, and then wanes rapidly. It is reinduced with booster injections. Thus, the kinetics mimic a typical IgM response (perhaps explaining why previous investigators sought to raise IgM monoclonal antibodies rather than IgG monoclonal antibodies).

e. The fraction of IgG that binds LPS is not measured by ELISA assays using *E. coli* J5 LPS as an antigen. Accordingly, cross-reactive monoclonal antibodies could not be screened for using this assay.

This information should be helpful to generate IgG monoclonal antibodies that would cross-react.

III. Significance

We believe that our work over the last year is important on several fronts. First, we have studied two commercial monoclonal antibodies (HA-1A and E5) that were widely promoted to bind and neutralize endotoxin. Using the reagents and techniques in the grant we found that the binding of each was slight and that neither neutralized LPS. These findings are consistent with the results of the clinical trials, which seem to indicate to us a marginal clinical effect. Each of these agents came close to being used widely in the United States for the treatment of gram negative sepsis at great cost. Trials are underway for HA-1A for a subgroup of the first trial in which there seemed to be considerable positive effect: patients who have sepsis and shock. Hopefully, this trial will confirm this effect. If so, our data suggest that HA-1A would be working by a different mechanism than neutralization, such as by clearing LPS from the bloodstream. This hypothesis remains to be tested.

As noted above, we have made considerable progress in understanding the interactions of tritiated LPS and polyclonal antiserum to rough mutants, such as *E. coli* J5. The protection offered by this antiserum is the experimental basis upon which anti-core and anti-lipid A monoclonal antibodies are based. Our findings that normal serum is needed for the binding, that IgG is as or more active than IgM, that the IgG that binds is a cryoglobulin, and that IgG formed early after the primary immunization binds best all provide information needed to develop cross-reactive monoclonal antibodies. The factor in normal serum that facilitates the binding remains unknown.

We feel that our results regarding the known LPS binding proteins that we have studied are very significant. Although we have been working on this area for only a year, we believe that we have identified the LPS binding site of all three proteins. Preliminary data suggest that this site may be homologous, at least antigenically, between all three. Two of the three proteins (used in their entirety) are being developed for the treatment of gram negative sepsis.

Identification of the binding site should permit development of agents that have higher activity and that are smaller and therefore not immunogenic. An exciting possibility may be to couple a synthetic peptide containing the active site to IgG or purified Fc fragments of IgG to make a synthetic hybrid molecule that binds and clears LPS. We have prepared such a reagent and finds that it converts normal IgG into IgG that binds LPS with high affinity. The identification of the LPS binding sites should have two other ramifications. First, since LPS binds to LBP in serum and the LPS-LBP complex binds to the CD14 receptor of macrophages with a resultant increase in TNF production, a synthetic peptide mimicking the LPS binding site should compete with binding for LBP and thus block its action. Early experiments suggest that this is the case. Second, it appears that the active site has been strongly conserved over time. The family of synthetic peptides that we have developed should provide excellent reagents to probe the metabolism and physiological roles of the natural LPS binding proteins. This information, in turn, should be helpful in the development of therapeutic agents.

IV. Publications over the last year

1. Warren HS, Glennon M, de Deckker FA, Tello D. Role of Normal Serum in the binding of Lipopolysaccharide to IgG fractions from rabbit antisera to Escherichia coli J5 and other gram-negative bacteria. J. Infect. Dis. 1991; 163:1256-1266.
2. Warren HS, Glennon ML, Wainwright N, Amato SF, Black KM, Kirsch SJ, Riveau GR, Whyte RI, Zapol WM, Novitsky TJ. Binding and neutralization of endotoxin by Limulus antilipopolysaccharide factor. Infect Immun 1992; 60:2506-2513
3. Warren HS, Danner RL, Munford RS. Anti-endotoxin monoclonal antibodies. N. Eng J Med 1992; 326:1152-1157.
4. Chen TY, Warren HS, Greene BA, Black K, Frostell CG, Robinson DR, Zapol WM. Protective effects of anti-O polysaccharide and anti-lipid A monoclonal antibodies on the ovine pulmonary circulation. J. App. Physiol. (In press).
5. Warren HS, Amato SF, Fitting C, Black K, Loissette P, Pasternack MS, Cavallion J-M. Assessment of ability of murine and human anti-lipid A monoclonal antibodies to bind and neutralize lipopolysaccharide. J. Exp. Med. (In Press)

Book chapters over the last year

1. Warren HS, Burke JF. Infection of Burn Wounds: Evaluation and Management. In: Swartz MN and Remington JS, ed. Current Clinical Topics in Infectious Disease, 11th ed. Blackwell Scientific Press. 1991.
2. Warren HS. Adjuvants. In: Roitt IM and Delves PJ, eds. Encyclopedia of Immunology. Philadelphia: W.B. Saunders Co. 1992.
3. Lynfield R, Warren HS. Antimicrobial treatment of sepsis in the pediatric intensive care unit. In: Todres D and Fugate J, eds. A practice of Pediatric Intensive Care, Little, Brown and Co, Boston. (In press).

Material for additional articles

[REDACTED]

We have completed the experiments for several additional articles and it will be important to get these results written up and submitted in the near future. Anticipated articles are:

1. Our newer results regarding the role of normal serum elements in the immunoprecipitation of LPS complexes by IgG and IgM.
2. The results of the screening pilot project that revealed that there are some donated human plasma units that contain high IgG titers directed to multiple clinically important gram negative strains. (The data for this article is outlined in the July 12, 1991 trimester report).
3. An article on the detection of LPS in tissues using LAL and ELISA techniques.
4. An article comparing the kinetics and tissue localization of LPS in tissues after endotoxemia with bacteremia (Submitted).
5. The work involving the mapping of the LPS binding site of LALF described above.

V. Future goals

We are in the last 2-3 months of support from our initial three year grant. One goal will be to write up and submit our backlog of results. Other goals that we hope to accomplish in the next several years are:

1. Development of therapeutic approaches based on our identification of the LPS binding sites of several proteins using our panel of synthetic peptides.

One peptide has already been found to be protective. We hope to use the peptides to assess the importance of the clearance of LPS vs its neutralization using the peptide families. We hope to create several secondary constructs containing the peptides including multiple copies of the peptides linked in "comb" and "snowflake" configurations, as well as coupling the peptides to IgG. We will then compare the ability of these reagents to bind, neutralize, and clear LPS in animal models.

2. Study of each of the peptides and the secondary structures to bind, kill, and opsonize bacteria.
3. Study of the ability of each of the peptides to block the known functions of each of the parent proteins.
4. Study of the ability of antiserum to each of the active sites to block the functions of the parent proteins.
5. Identification and characterization of the normal serum element that permits binding of tritiated LPS to E. coli J5. Identification of its LPS binding site if there is one. Eventual attempt at developing cross-reactive monoclonal antibodies based on a screening assay containing this factor.
6. Assessment of the protective efficacy of plasma screened for high titers to multiple strains of gram negative bacteria

TABLE 1

LPS BINDING ACTIVITY (ng)												MEAN ±1-S.D.
#1	#2	#3	#4	#5	#6							
LIMULUS ANTI-LPS FACTOR												
30	40	50	58									
MM4- G H E C H Y R I M P T V K R L K W K Y K G K F W C P S U T S I -COOH				63	63	63	63	125	31		62.5 30.8	
				>50000	>50000	>50000	>50000					
				>50000	>50000	>50000	>50000					
				>50000	>50000	>50000	>50000					
				125	125	125	125				125.0 0.0	
				125	125	125	125				125.0 0.0	
				250	125	250	250				218.8 62.5	
				500	250	250	250				312.5 125.0	
				250	125	250	250				218.8 62.5	
				250	125	250	250	250	250		229.2 51.0	
				50000	12500	25000	12500	25000			25000.0 15309.3	
				6250	12500	3125	12500	12500			9375.0 4419.4	
				12500	12500	6125	12500				10906.3 3187.5	
				>50000	>50000	>50000	>50000	25000				

TABLE 2

HUMAN LBP	LPS BINDING ACTIVITY (ng)					
	#1	#2	#3	#4	MEAN	± SD
NH ₂ -A N P G L V A R I T D K G L Q Y A A Q E G L L A L O S E L L R I T L P D F T G D L R I P H V G R G R Y E F N S L M I N S 10 20 30 40 50 60 A N P G L V A R I T D K G L Q Y A A Q E G L L A L O S E L L O S E L L R I T L P D F T G D L R I P H V G R G R Y E F N S 781 1500 390 1000 722.5 661.8 peak#1 3125 3125 781 1562 1367.0 1334.3 peak#2 6000 1000 390 781 292.8 373.8						
I P H V G R G R Y E F N S L M I N S C E L L M S A L R P V P G G G L S L S I S D S S I R V Q G R U K V R K S F F K L G G -C O O H 50 60 70 80 90 Y E F H S L M I N S C E L L M S A L R P V P G G G L S L S I L S L S I S D S S I R V Q G R U K V R K S F F K L G G C R V Q G R U K V R K S F F K L G G C	6250	77	781	781	390.5	450.9
	250	250	250	62	140.5	128.9
	500	250	250	250	187.5	125.0

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ASSESSMENT OF ABILITY OF MURINE AND HUMAN ANTI-LIPID A MONOCLONAL ANTIBODIES TO
BIND AND NEUTRALIZE LIPOPOLYSACCHARIDE

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Condensed title: Ability of anti-lipid A Mabs to bind and neutralize LPS

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ABSTRACT

The use of monoclonal antibodies directed to lipid A for the therapy of gram-negative sepsis is controversial. In an attempt to understand their biologic basis of action, we utilized a fluid-phase radioimmunoassay to measure binding between bacterial lipopolysaccharide and two IgM monoclonal antibodies directed to lipid A that are being evaluated for the treatment of gram-negative bacterial sepsis. Both antibodies bound ^3H -LPS prepared from multiple strains of gram negative bacteria when large excesses of antibody were utilized, although binding was modest and only slightly greater than control preparations. We also studied the ability of each anti-lipid A antibody to neutralize some of the biological effects of lipopolysaccharide *in vitro*. Despite large molar excesses, neither antibody neutralized lipopolysaccharide as assessed by the Limulus lysate test, by a mitogenic assay for murine splenocytes, or by the production of cytokines IL-1, IL-6, or TNF from human monocytes in culture medium or in whole blood. Our experiments do not support the hypothesis that either of these anti-lipid A monoclonal antibodies function by neutralizing the toxic effects of LPS.

INTRODUCTION

In 1968, Chedid et al. reported that the passive infusion of antiserum raised to killed rough mutant bacteria protected mice against challenge with heterologous gram negative bacteria (1). Rough mutant bacteria are unable to incorporate O-polysaccharide onto the glycolipid of LPS, and therefore expose

the deeper core structures of endotoxin on their surface. These authors proposed that a few antibodies or serum factors specific for the endotoxin core could "have the capability of coping, like masterkeys, with a very wide range of infections due to serologically unrelated organisms" (1). Over the next two decades several laboratories studied the ability of antisera raised to two bacterial mutants, E. coli J5 and S. minnesota Re595, to protect in animal models of endotoxic shock. Most (1-7), but not all (8,9), of these studies confirmed that passively transferred antisera raised to these organisms protect against challenge with heterologous LPS in animal models. A clinical trial indicated that polyclonal antiserum raised to E. coli J5 was therapeutic for patients with gram negative sepsis (10). Two lines of evidence suggested that the protective element in these antisera was immunoglobulin directed against common epitope(s). First, purified immunoglobulin fractions prepared from the polyclonal antisera protected in animal models (4,11,12). Second, the protective activity in the antisera could be absorbed with the rough mutant LPS or bacteria, but not with control heterologous LPS or bacteria (1,5,6,13,14). The concept evolved that some of the immunoglobulin in these antisera cross-reacted with LPS from multiple gram negative strains, and therefore was able to "cross-protect" against heterologous strains. Cross-reactive immunoglobulin was hypothesized to protect by neutralizing the toxic effects of endotoxin (10).

Several investigators have generated monoclonal antibodies to structures on the core glycolipid of LPS (15-20). Two of these, HA-1A (Centocor, Malvern, PA) and E5 (XOMA, Berkeley, CA), have been studied in human trials which enrolled patients with suspected gram negative sepsis (16,21,22). HA-1A is a human IgM monoclonal antibody which binds to the lipid A (15). This antibody has been reported to protect in some animal models (15,23), although in other models the protection has been only modest (24,25). The protective efficacy of HA-1A for

gram negative sepsis has been studied in a single double blind, randomized, placebo-controlled clinical trial (21). In this study, administration of HA-1A was associated with decreased 28 day all-cause mortality in a subgroup of patients that had gram negative bacteremia. HA-1A is licensed for use as a therapy for gram-negative sepsis in some countries in Europe. E5 is a murine IgM monoclonal antibody which also binds to lipid A (16). There are few data available regarding the ability of E5 to protect in animal models. Two articles suggest that it provides slight protection against endotoxin (26) or bacterial challenge (27). E5 has been evaluated in two clinical trials. In the first trial, administration of E5 was associated with increased survival in a subset of patients with gram negative sepsis who were not in refractory shock (16). In the second trial there was apparently no improved survival in patients with documented gram negative sepsis, although these results have been presented in abstract form only (22).

Because a proposed mechanism of action of these two antibodies is to bind and neutralize lipopolysaccharide, we studied the ability of each to bind the LPS from multiple clinical strains of gram negative bacteria. The results of solid phase immunoassays can be difficult to interpret because of non-specific interactions and the amphipathic nature of LPS. Accordingly, we used a fluid-phase radioimmunoassay to measure antibody-LPS binding. We also evaluated the ability of each anti-lipid A antibody to neutralize the effects of LPS in several *in vitro* assays of endotoxin bioactivity. We found that both HA-1A and E5 bound slightly to LPS from multiple smooth strains of gram negative bacteria when large excesses of antibody were utilized. However, neither antibody neutralized LPS as assessed by the Limulus lysate test, by a mitogenic assay for murine splenocytes, or by the production of the cytokines IL-1, IL-6, or TNF from human monocytes in culture medium or in whole blood. Our experiments do

not support the hypothesis that either of these two anti-lipid A monoclonal antibodies function by binding and neutralizing the toxic effects of LPS.

METHODS

Lipopolysaccharides

Unlabeled LPS from S. typhimurium, K. pneumoniae, S. marcescens, E. coli J5, and E. coli K12,D31m4 (Re) were purchased from List Co. (Campbell, CA). Unlabeled LPS from E. coli O113 was prepared by the hot phenol method as described by Rudbach (28). Unlabeled LPS from N. meningitidis was the kind gift of Martine Caroff (UA Centre National de la Recherche Scientifique 1116, Orsay, France).

Cultures of E. coli O111:H4, E. coli O18, and E. coli J5 were the kind gifts of Dr. David Morrison (University of Kansas Medical Center, Kansas City, Kansas), Dr. George Siber (Dana Farber Cancer Institute, Boston, MA), and Dr. Jerald Sadoff (Walter Reed Army Institute of Research, Washington, DC, respectively). Cultures of E. coli strains O1, O2, O4, O6, O7, O8, O16, O25, and O75 were the kind gifts of Dr. Alan Cross (Walter Reed Army Institute of Research, Washington, D.C.).

Biosynthetically radiolabeled strains of E. coli strains (except for E. coli J5) were prepared by growing the organisms in the presence of ^3H -acetate followed by hot phenol extraction as previously described (29). Briefly, we grew cultures of each organism to an optical density of 0.9 at 540 nm (with a path length of 1.0 cm) in broth containing, per liter, 22.5 g of yeast extract, 11 g peptone, 4 g of monobasic potassium phosphate, 16.8 g of dibasic potassium

phosphate, and 10 g of glucose, in the presence of 10 mCi of ^3H -acetate per 100 ml broth. The cells were chilled and washed three times in saline, and the LPS was extracted by the hot phenol method (30). The preparations were then treated with DNase and RNase and then with pronase (Sigma chemical Co, St. Louis, Mo) according to the method of Romeo (31). The concentrations of LPS were estimated by a spectrophotometric LAL gelation assay utilizing an E. coli O113 LPS standard containing 10 endotoxin units/ng (lot 20; Associates of Cape Cod, Falmouth, MA) (32). These results were similar to those obtained by weight. Solutions of ^3H -LPS were adjusted to 1 $\mu\text{g}/\text{ml}$ and cpm/ μg were calculated by counting a 0.4-ml volume combined with 4.5 ml of optiflor scintillation fluid (Packard, Downers Grove, IL). The different LPS contained the following cpm/ μg : E. coli O1- 7,110, E. coli O2- 13,810, E. coli O4- 10,490 cpm/ μg , E. coli O6- 7,200 cpm/ μg , E. coli O7- 12,150, E. coli O8- 10,990, E. coli O16- 17,100, E. coli O18- 6,150 cpm/ μg , E. coli O25- 11,040 cpm/ μg , E. coli O75- 4,700 cpm/ μg , E. coli O111:B4- 4,040 cpm/ μg . Greater than 99% of each radiolabeled LPS was demonstrated to remain in the water phase after 1:1 ether-water extraction at pH 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of each LPS resulted in a regularly spaced band pattern typical of LPS when stained with silver. Similar regularly spaced band patterns were obtained when the gels were analyzed by autoradiography.

Radiolabeled LPS from E. coli J5 was made by growing the organisms in broth containing ^3H -acetate as described above and extracting the LPS as described by Galanos (33). This LPS contained 22,000 cpm/ μg .

Antibodies and controls

The human anti-lipid A IgM monoclonal antibody (MA-1A, Centoxin) used in

the study was from Centocor B.V. (Leiden, Netherlands). It was supplied as a 5 mg/ml solution containing 5% human serum albumin. Murine anti-lipid A IgM monoclonal antibody (E5) was the kind gift of Dr. Robert Rubin (Infectious Disease Unit, Massachusetts General Hospital, Boston, MA). It was supplied as a 2 mg/ml solution in phosphate buffered saline, pH 7.3. Murine IgG2a monoclonal antibody directed to the O-polysaccharide of E. coli O111:B4 was the kind gift of Dr. Matthew Pollack (Uniformed Health Services University and Health Sciences, Bethesda, MD). Human myeloma IgM and murine polyclonal IgM were purchased from Rockland Inc. (Gilbertsville, PA). Polyclonal human IgM was purchased from Organon Teknika Corp (West Chester, PA). Polymyxin B used in the mitogenic experiments and the cytokine experiments were obtained from Pfipharmes (New York, NY).

Fluid-phase radioimmunoassay

Radiolabeled LPS was incubated in dilutions of each monoclonal antibody or controls at 37°C. Complexes of ³H-LPS bound to protein were then separated from free ³H-LPS by precipitation in half-saturated ammonium sulfate according to the method of Farr (34).

Specifically, 5 ug/ml of each smooth LPS tested was incubated in 150 ul of dilutions of the monoclonal antibody to be tested in .01M phosphate buffered saline, pH 7.3 for 120 minutes at 37°C in a 1.5ml microcentrifuge tube. Controls were human serum albumin (HSA), polyclonal human IgM, human myeloma IgM, polyclonal murine IgM, and a murine IgG Mab directed to the O polysaccharide of E. coli O111:B4. Following incubation, the solution was cooled on ice for 15 minutes. An equal volume of iced saturated ammonium sulfate was then added drop wise and the solution was allowed to sit at 4°C for another 15

minutes. The tubes were next centrifuged at 12,000 x g for 15 minutes. Supernatants were carefully aspirated, and pellets were washed twice with 50% ammonium sulfate and resuspended in 300 ul of PBS. The quantity of LPS in the supernatants and pellets were assessed by counting 0.4 ml of a 1:7 dilution of each combined with 4.5 ml of optiflor scintillation fluid (Packard, Downers Grove, IL). Quenching was minimal and was corrected by using the internal standard method. Greater than 99% of the HA-1A was documented to be in the pellet following precipitation with 50% ammonium sulfate as assessed by a capture IgM ELISA technique. Greater than 80% of E5 was found in the pellet following precipitation as assessed by a protein assay using bicinchoninic acid (Pierce, Rockford, IL). Less than 5% of each smooth LPS tested precipitated in PBS alone. However, greater than 90% of the ^3H -LPS from rough mutant E. coli J5 precipitated in PBS-HSA alone, so that we were unable to test this LPS in the system. For most of the assays, recovery of radioactivity was greater than 85% using this assay. At low protein concentrations (less than 50 ug/ml) recovery was sometimes less, which we attributed to LPS binding to the walls of the tube. All assays were performed in duplicate and the results are given as means. Results were calculated as percentage recovered cpm as determined by the formula: (cpm in the pellet/cpm recovered) x 100, expressed to the nearest whole percentage point. Results are given as the mean of at least two experiments.

Limulus amoebocyte lysate (LAL) assay

A spectrophotometric Limulus lysate assay was utilized as previously described (35). Briefly, 50 ul of a solution of 20 ug/ml of each monoclonal antibody in pyrogen free saline were incubated with 50 ul of dilutions of LPS for 30 min at 37°C in a 96 well microtiter plate. One hundred ul of LAL were then added and the plate was incubated at 37°C for an additional 60 minutes.

Coagulation of the LAL was measured spectrophotometrically at OD₄₀₅ in an automated ELISA reader. Coagulation of the LAL in the presence of HA-1A or E5 was compared to coagulation of the LAL in saline alone.

Mitogenic assay

The mitogenic assay was performed essentially as described by Jacobs (36). Briefly, dilutions of LPS were preincubated in the presence of 625 ug/ml monoclonal antibody or control or 50 ug/ml polymyxin B for 2 hours at 37°C. This solution was then diluted in RPMI media supplemented with antibiotics containing 0.01 M Hepes and 10% fetal calf serum and incubated in a volume of 200 ul for 48 hours with 5×10^6 spleen cells from Balb/C mice. The final antibody or HSA control concentrations in culture were 25 ug/well and the final polymyxin B concentration in culture was 2 ug/well. One microcurie of ³H-thymidine was next added to each well and cells were incubated for an additional 16 hours. Incorporated radioactivity was measured using a cell harvester (Cambridge Technology, Watertown, MA) to disrupt the cells followed by scintillation counting. Each assay was done in quadruplicate, and the results are given as the mean.

Cytokine Assays

Preparation and treatment of human monocytes.

Peripheral blood mononuclear cells were obtained by centrifugation on Ficoll (MSL; Eurobio, France) of 1:2 diluted heparinized venous blood from healthy adult volunteers. Monocytes were selected by allowing the mononuclear cells to adhere to plastic culture dishes (24 wells; Nunc, Roskilde, Denmark) in the absence of serum (37). More than 85% of the adherent cells were monocytes,

as assessed by morphological analysis by phase-contrast microscopy, histochemical staining for nonspecific esterase activity (38), and indirect immunofluorescence staining using anti-lymphocyte antibodies, OKT11 (Ortho Diagnostics, Inc., Raritan, N.J.) and IOB1 (Immunotech, Marseille, France). Human mononuclear adherent cells (5×10^5 nonspecific esterase-positive cells per well), cultured in RPMI 1640 medium (GIBCO) supplemented with 100 IU of penicillin per ml and 100 μ g of streptomycin per ml, were incubated for 24 h in the presence of free LPS, or in the presence of LPS which had been previously incubated with dilutions of anti-lipid A antibodies or 2 μ g/ml polymyxin B for 1 hour at 37°C in culture medium containing 3.3% de complemented normal human serum. These solutions were then added to the cells so that the final serum concentration in all cultures was 0.2%. Final reaction mixtures contained the indicated amounts of antibody and LPS in a volume of 0.5 ml per well. Culture supernatants were collected and centrifuged at 3,000 X g for 15 min. and assayed for cytokines. IL-1 found in the supernatant will be referred to as IL-1 or released IL-1. The adherent cells were then lysed by three freeze-thaw cycles in 0.5 ml of fresh RPMI 1640 medium and the lysates were centrifuged at 3,000 X g for 15 min. The IL-1 found in these supernatants will be referred to as cell-associated IL-1.

Whole blood cytokine assays

The induction of cytokines by LPS from whole blood was measured as described (39). Briefly, 500 μ l blood diluted 1:5 in RPMI-1640 culture medium per well was cultured in the presence of free LPS, or LPS that had been preincubated with antibodies or polymyxin B as above. Final reaction mixtures contained the indicated amounts of antibody and LPS. Cytokines were assayed following 24 hours of culture.

TNF assay.

TNF α RIA. An RIA specific for TNF α was performed according to Munoz et al. (37) with minor modifications. Briefly, on day 1, 100 μ l of a rabbit anti-TNF α antiserum (a kind gift of Catherine Rougeot, Institut Pasteur) diluted 1:8,200 to precipitate 35% of the radiolabeled TNF was added to 100 μ l of standards or samples. To determine the nonspecific binding, 100 μ l of BSA buffer was added to a tube instead of sample. Then 300 μ l of BSA buffer was added to each tube. After vortexing, the tubes were incubated for 24h at room temperature. Standards of TNF α (Rhone Poulenc, France) containing 0, 40, 80, 150, 300, 600, 1,2500, 2,500, 5,000, and 10,000 pg/ml were employed. On day 2, 100 μ l of a solution of ^{125} I-TNF α (30 μ Cl/ μ g, New England Nuclear, Boston, MA) containing = 10,000 cpm was added to each tube. The tubes were vortexed and incubated at room temperature for 24h. On day 3, 500 μ l of BSA buffer containing 6% of polyethylene glycol 8000 (Sigma Chemical Co.), 1% of horse anti-rabbit IgG and 0.1% of normal rabbit serum were added. The tubes were vortexed and incubated for 2 h at 4°C. The tubes were then centrifuged at 1,500 g for 15 min at room temperature. Thereafter, the supernatants were discarded and the tubes were kept inverted for 30 min and drained on absorbent paper. Tubes were counted in a gamma counter and the value for nonspecific binding was subtracted. All standards and samples were expressed as percentage of the standard containing no TNF α (zero standard). The concentrations of TNF α in pg/ml on a logarithmic x-axis were plotted against the binding percentage on a logarithmic y-axis. The standard curve obtained was used to determine the concentrations of TNF α in samples. The detection limit was 70 pg/ml (set binding of 95% of zero standard).

IL-1 assay.

IL-1 β RIA. A similar RIA protocol as that used for TNF α measurement was

employed to determine concentrations of IL-1 β . Standard IL-1 β was obtained from Rhone Poulenc (France). Rabbit anti-IL-1 β antiserum (1:150) was purchased from Endogen (Boston, MA), and ^{125}I -IL-1 β (126-253 $\mu\text{Ci}/\mu\text{g}$) was obtained from New England Nuclear (Boston, MA). On day 3, 500 μl of BSA buffer containing 6% polyethylene glycol 8000, 1% of sheep anti-rabbit IgG (Sigma Chemical Co.) and 0.05% normal rabbit serum were added to each tube. Determination of IL-1 β concentrations in plasma and samples were calculated as described above. The detection limit was 70 pg/ml.

IL-1 α ELISA. The IL-1 α concentrations were determined by ELISA using two anti-IL-1 α monoclonal antibodies as described (37). Briefly, on day 1, Luxlon ELISA microtiter plates were coated with 100 μl of monoclonal mouse IgG1 anti-rhIL-1 α (10 $\mu\text{g}/\text{ml}$ in carbonate buffer) and incubated 2 h at 37°C. The plates were washed three times with 0.1% Tween-20 PBS. Standards (0, 10, 30, 300, 1,000, 3,000, and 10,000 pg/ml rhIL-1 α) or cell supernatant or cell lysate samples diluted in 1% BSA, 0.1% Tween-20 PBS were added to coated wells and incubated overnight at 4°C. On day 2, the plates were washed three times and 100 μl of the second monoclonal mouse anti-rhIL-1 α (IgG2b) (1:2,000 in BSA/Tween/PBS) were added into each well. Plates were incubated for 3 h at 37°C. After washing, 100 μl of peroxidase conjugated anti-mouse IgG2b (1:5,000) (Southern Biotechnical Associates, Inc., Birmingham, AL) were added to each well and the plates were left for 1 h at 37°C. After washing, enzymatic activity was detected with a phosphate citrate buffer containing 1 mg/ml O-phenylenediamine dihydroxychloride (Sigma Chemical Co.) and hydrogen peroxide (0.06%). The reaction was stopped with 50 μl of 3N HCl and the absorbance was read at 492 nm in a microplate reader (Titertek multiskan MC340; Flow laboratories, Inc., McLean, VA). The levels of IL-1 α in the samples were calculated by reference to the standard curve. The detection limit of IL-1 α was 30 pg/ml.

IL-6 assay.

IL-6 Bioassay. IL-6 activity was determined as described (37) by using the specific 7TD1 IL-6-dependent cell line (40) kindly provided by Dr. J. Van Snick (Ludwig Institute for Cancer Research, Brussels). Cells were cultured at a density of 1,200 cells/well (96-well multidish plates, Falcon Labware, Oxnard, CA) in 100 μ l of RPMI medium supplemented with antibiotics, 2-mercaptoethanol (5×10^{-5} M) and 10% FCS, in the presence of serial dilutions of cell supernatants. After 4 d of culture at 37°C, the proliferation was monitored by a dye method (55). Briefly, 125 μ g of tetrazolium salts (MTT) were added to each well and after 1-2 h of incubation at 37°C, the reaction was stopped with 100 μ l/well of an extraction buffer (20% SDS, 50% DMF, 2.5% 1.0N HCl, 2.5% of 80% acetic acid, pH 7.4). After overnight incubation at 37°C, optical densities were measured at 540 nm using an automated microELISA autoreader. One unit of IL-6 corresponds to half-maximum growth of the hybridoma cells. IL-6 activity detected in supernatants of LPS-stimulated monocytes and in plasma was completely abolished by the addition of 10 μ g rabbit polyclonal anti-human IL-6 antibodies (Genzyme Corp., Boston, MA).

RESULTS

Binding of HA-1A and E5 to ^{3} H-LPS

At a concentration of 200 μ g/ml, HA-1A consistently bound slightly more radiolabeled LPS in comparison to controls (Table 1). In two instances (E. coli O1 and E. coli O2) substantial quantities of radiolabeled LPS were bound by HA-

1A, but these strains were bound considerably by control polyclonal human and mouse IgM/HSA as well. In contrast, there was little or no difference in the amount of LPS bound by E5 compared to controls (Table 1). We varied the concentration of monoclonal antibodies in order to compare binding with the O-specific IgG antibody and to see if higher concentration of anti-lipid A Mabs would be more efficient at binding. These results are shown in Figure 1. At very high concentrations (1.6 mg/ml), E5 bound up to 30% of the LPS from certain strains, which was slightly higher than the polyclonal IgM control in this series of experiments, while HA-1A bound somewhat less. As expected, the O-specific IgG monoclonal antibody bound homologous LPS efficiently at low concentrations (half-maximal binding at 5-10 ug/ml).

Neutralization of LPS as assessed by Limulus lysate

A representative experiment assessing the ability of HA-1A and E5 to neutralize LPS in the Limulus lysate assay is shown in Figure 2 using LPS from rough mutant E. coli J5. Neither antibody inhibited the activity of LPS over a wide range of LPS concentrations. In additional experiments (not shown) LPS from smooth strains E. coli O18, E. coli O113, and E. coli O111:B4 and rough strain N. meningitidis were tested. No neutralization of LPS by either monoclonal antibody was observed. Each experiment was repeated three times for each LPS and each antibody.

Neutralization of LPS as assessed by splenocyte mitogenic assay

LPS from smooth strains E. coli O18 and E. coli O113, and rough strains E. coli J5 and N. meningitidis were tested. Neither HA-1A or E5 inhibited the

activity of any of the LPS tested for mitogenicity. In contrast, polymyxin B abrogated the mitogenic effects of LPS over a wide range of LPS concentrations. A representative experiment is shown in figure 3. Each experiment was performed three times for each anti-lipid A antibody.

Neutralization of LPS as assessed by induction of cytokines from monocytes

In this series of experiments, dilutions of LPS were preincubated with differing concentrations of HA-1A and E5 and then exposed to cultured human monocytes or incubated in whole blood. The induction of TNF, IL-1, and IL-6 by these mixtures were then assessed as described in Methods. LPS from smooth strains E. coli O111:B4 and E. coli O18 and rough strains E. coli K12,D31m4 (Re), E. coli J5, and N. meningitidis were tested. HA-1A and E5 had no significant effect on the induction of TNF, IL-1, or IL-6 in the two types of assay system. Representative experiments for HA-1A for the adherent cell system (n=5) and the whole blood system (n=7) are shown in figures 4 and 5 respectively. Representative experiments for E5 (n=2, adherent cells; n=4, whole blood) are shown in fig. 6.

DISCUSSION

These studies demonstrate that anti-lipid A monoclonal antibodies HA-1A and E5 bind only weakly to LPS from multiple clinically relevant smooth gram negative bacteria, and are unable to neutralize the biological effects of LPS in several *in vitro* assays. Our findings do not support the hypothesis that either of these antibodies is able to protect patients with gram negative sepsis by

binding to lipid A and blocking the toxic effects of endotoxin in the bloodstream.

Each of these monoclonal antibodies have been described to bind to lipid A and LPS as assessed by ELISA (15,41), although a more recent abstract suggested that HA-1A binds only slightly to smooth LPS using this technique (42). Since solid phase assays using LPS are difficult to interpret because of non-specific binding to the solid phase matrix, we utilized a fluid-phase radioimmunoassay to measure antibody-LPS binding. We found that both HA-1A and E5 bound to radiolabeled smooth LPS compared to irrelevant IgM controls, but that it was necessary to utilize very high concentrations of IgM to see the effect. We were unable to achieve half-maximal binding of any of the bacterial strains tested even with 1.6 mg/ml HA-1A or E5, whereas half-maximal binding of E. coli O111:B4 LPS was achieved at 5-10 ug/ml of an IgG monoclonal antibody specific for the O-polysaccharide of this LPS. The radioimmunoassay that we utilized was initially described by Farr (34), and depends upon the precipitation of antigen-antibody complexes in 50% ammonium sulfate while free antigen remains in solution. We were not able to assess binding of either antibody to rough LPS from E. coli J5 because we found that greater than 90% of this LPS is precipitated by 50% ammonium sulfate. Since high concentrations of HA-1A and E5 were needed to precipitate LPS, we cannot exclude the possibility that some or all of the binding that we measured at high antibody concentrations is non-specific. It has been suggested that anti-core glycolipid antibodies may interact with LPS in a non-specific manner through hydrophobic interactions (43). Indeed, we found that some of the control antibody preparations bound appreciable quantities of LPS at high concentrations.

There is no previously published information on the ability of these two

anti-lipid A antibodies to neutralize the biological effects of LPS. We investigated the ability of the Mabs to inhibit LPS-induced activation in the Limulus lysate assay and proliferation of murine splenocytes. Lipid A plays a major role in each of these assays (44). In addition, we studied the capacity of the Mabs to prevent LPS-induced monocyte production of IL-1, IL-6, and TNF. IL-1 and TNF are felt to be important in the pathogenesis of septic shock (45-50), and plasma levels of TNF and IL-6 have been reported to correlate with the outcome of patients with sepsis (51-54). Neither HA-1A or E5 was able to neutralize the effects of LPS in any of the assays we utilized, even when the antibodies were preincubated in excess with LPS from rough bacterial strains.

Our results need to be viewed with some caution. All of the experiments were performed with LPS that had been extracted chemically. It is possible that the monoclonal antibodies would bind better to bacterial membrane fragments. Recently, data have been presented in abstract form that HA-1A binds to gram negative bacteria that have been previously treated with antibiotics that are active against the bacterial cell wall (42). In addition, it is possible that we would have found more binding or some neutralization if we had used even higher concentrations of antibody in our assays. Nevertheless, the concentrations used in the binding assay are greater than physiologic, and the ratio of antibody to LPS in the neutralization assays that we used for the LAL, mitogenic assay, and cytokine assays were 10^6 , 10^3 , and 10^5 by weight. Although molar ratios would have approximately 90-fold less than this based on an estimated LPS and IgM molecular weights of 10,000 and 900,000, these ratios should have been more than adequate to see some evidence of neutralization, especially given that each antibody is pentameric. Since there are likely many mediators that contribute to the septic syndrome, it is also possible that the weak binding that we detected could affect mediators of sepsis other than the cytokines we measured,

and thereby exert a protective effect. Finally, the binding that we detected could lead to protection by mechanisms other than neutralization, such as by increasing the clearance of LPS.

Since E5 and HA-1A may be utilized clinically on a widespread basis, further work should be done on their mechanism(s) of action. This knowledge could lead to the development of an *in vitro* test that correlates with protective efficacy and might indicate which patients would most benefit from these expensive agents.

LEGENDS TO FIGURES

Figure 1. Binding of HA-1A (left panel) and E5 (right panel) to 5 ug/ml LPS from several smooth gram negative strains as a function of concentration. Background was subtracted from each point on the curves, and each curve is the mean of two experiments. Symbols are as follows: HA-1A and E5- solid symbols, solid lines; HSA control (left panel) and murine IgM control (right panel)- open symbols, dashed lines; E. coli O4- triangles; E. coli O23- circles; E. coli O75- squares; E. coli O111:B4- inverted triangles. A single experiment showing binding of an IgG monoclonal antibody directed to the O-polysaccharide of E. coli O111:B4 to the homologous LPS is also shown for comparison in each panel (inverted triangle, dotted lines. This antibody did not bind to LPS from other strains.

Figure 2. Effect of HA-1A (left) and E5 (right) on LPS-induced activation of Limulus amoebocyte lysate. Each monoclonal antibody was preincubated with the indicated concentrations of LPS from E. coli J5 before incubation with Limulus lysate. Optical density of reaction was measured after 1 hour.

Figure 3. Effect of HA-1A (left) and E5 (right) on LPS-induced mitogenic activity on splenocytes. Wells contained indicated amounts of LPS from E. coli O18 and 25 ug antibody or 2 ug polymyxin B or PBS.

Figure 4. Effect of HA-1A on LPS-induced stimulation of TNF α , cell-associated IL-1 α , and IL-1 β from adherent human monocytes. HA-1A had no effect on LPS-induced stimulation of IL-6 in this or other experiments. Wells contained indicated amounts of antibody and LPS or 1 ug polymyxin B.

Figure 5. Effect of HA-1A on LPS-induced stimulation of TNF α , IL-1 β , and IL-6 in whole blood assay. Wells contained indicated amounts of antibody and LPS or 1 ug polymyxin B.

Figure 6. Effect of E5 on LPS-induced stimulation of TNF α and IL-1 β from adherent human monocytes (panels labeled A), and in whole blood assay (panels labeled B). Wells contained indicated amounts of antibody and LPS or 1 ug polymyxin B.

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Table 1: Percentage of ^3H -LPS bound by different immunoglobulin preparations at $200\mu\text{g/ml}^\dagger$

^3H -LPS ($5\mu\text{g/ml}$)	PBS/HSA	Anti- <i>E. coli</i> O111:84/ HSA	Polyclonal H u m a n IgM/HSA	Human IgM myeloma/HSA	NA-1A/HSA	Polyclonal M o u s e IgM/HSA	ES/HSA
<i>E. coli</i> 01	3.8 ± 3.3	1.5 ± 0.7	9.7 ± 2.1	7.5 ± 3.5	26.2 ± 10.4	23.0 ± 0.0	11.0 ± 1.4
<i>E. coli</i> 02	3.5 ± 3.5	1.0 ± 0.0	42.0 ± 18.3	3.5 ± 2.1	60.7 ± 22.4	59.5 ± 3.5	8.0 ± 1.4
<i>E. coli</i> 04	2.5 ± 1.2	2.0 ± 1.4	5.0 ± 0.0	4.0 ± 1.4	15.1 ± 8.7	7.5 ± 3.5	5.0 ± 0.0
<i>E. coli</i> 06	1.2 ± 0.4	1.5 ± 0.7	2.0 ± 0.0	2.0 ± 0.0	7.5 ± 3	6.5 ± 6.4	4.0 ± 1.4
<i>E. coli</i> 07	1.3 ± 0.7	1.0 ± 0.0	1.5 ± 0.7	1.5 ± 0.7	10.5 ± 7.4		
<i>E. coli</i> 08	1.8 ± 1.1	1.0 ± 0.0	2.0 ± 1.4	3.0 ± 0.0	13.0 ± 3.6		
<i>E. coli</i> 018	1.4 ± 0.7	1.0 ± 0.0	2.0 ± 0.0	1.5 ± 0.7	9.8 ± 6.7		
<i>E. coli</i> 025	2.0 ± 1.2	1.0 ± 0.0	7.0 ± 6.2	2.5 ± 0.7	16.1 ± 9.2	3.0 ± 0.0	1.6 ± 0.5
<i>E. coli</i> 075	2.1 ± 1.7	1.5 ± 0.7	6.0 ± 4.3	1.5 ± 0.7	11.2 ± 5.5	5.5 ± 2.1	3.6 ± 2.1
<i>E. coli</i> O111:84	6.4 ± 5.8	65.0 ± 15.0	4.0 ± 1.4	5.0 ± 1.4	23.2 ± 8.5	3.0 ± 0.0	6.5 ± 7.8

† Each value represents mean $\pm$ standard deviation.

Fig 1

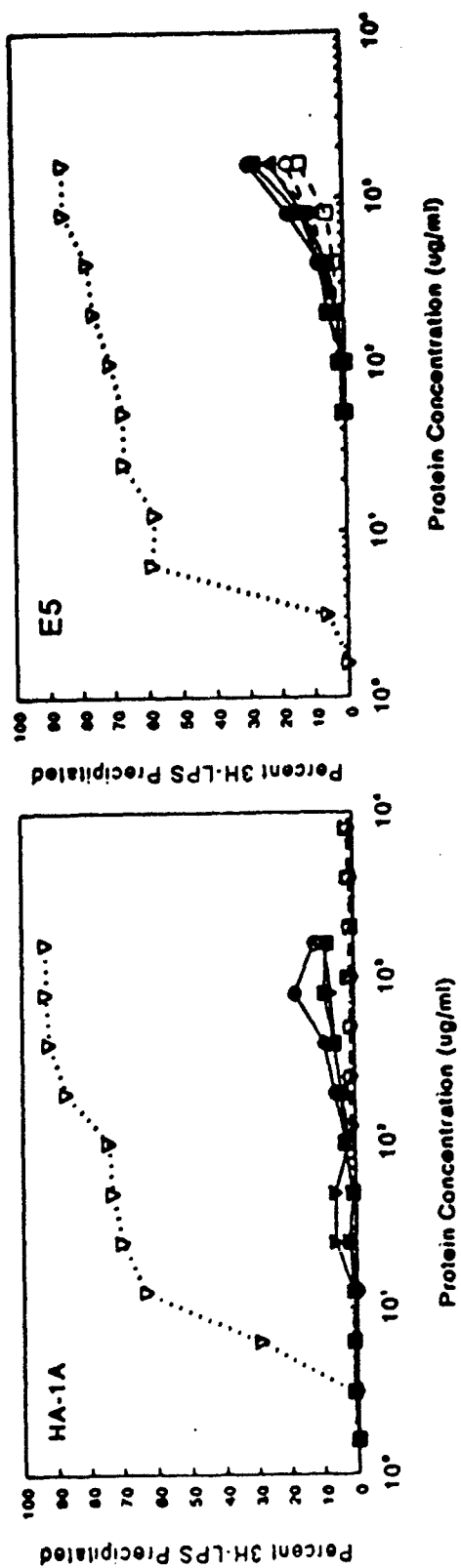


Fig 2

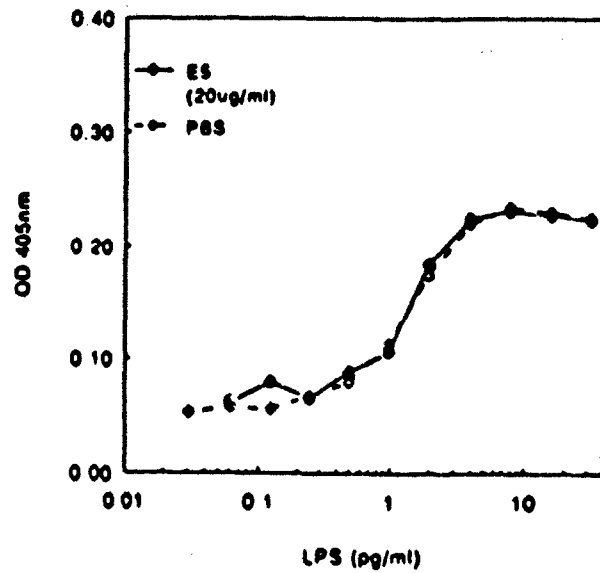
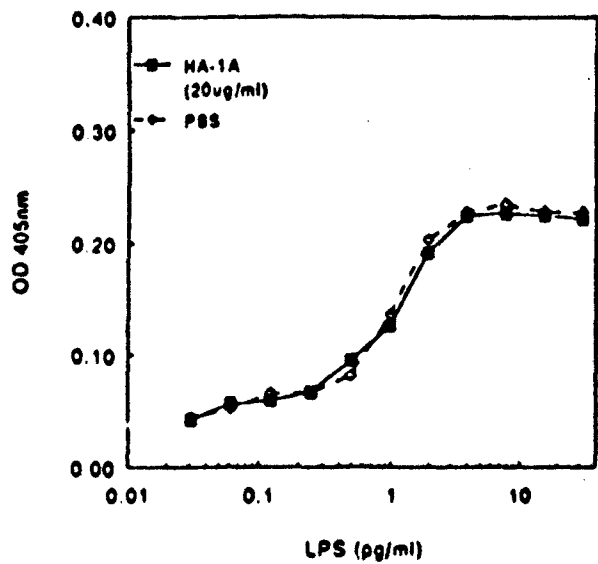


Fig 3

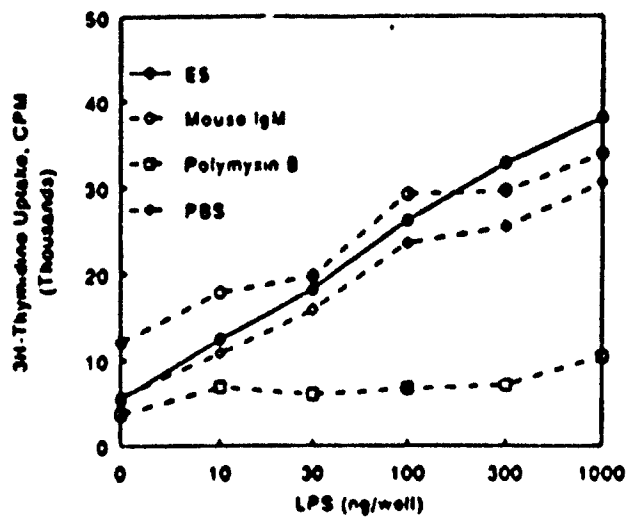
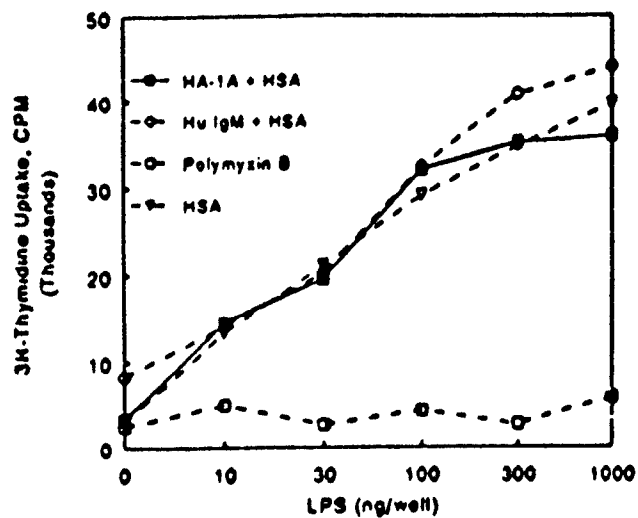


Fig 4

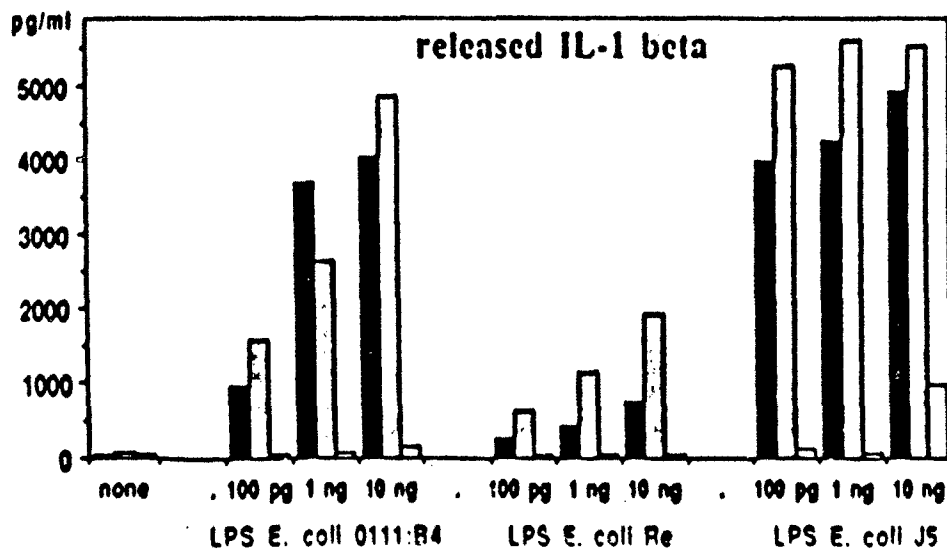
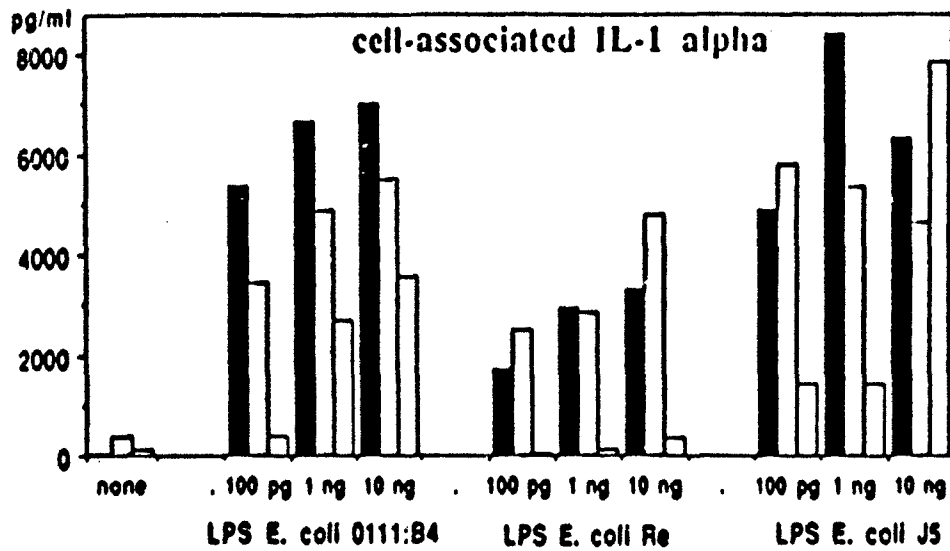
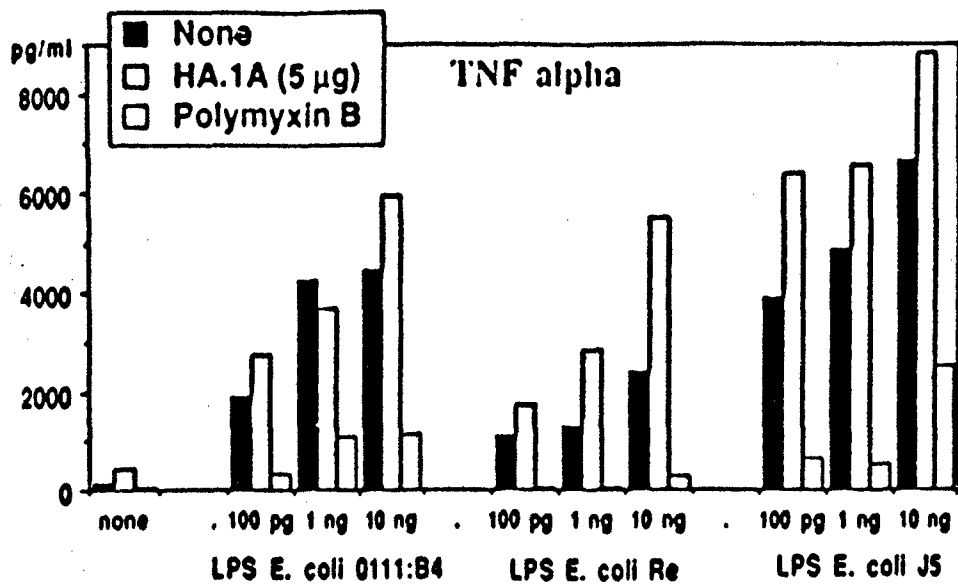
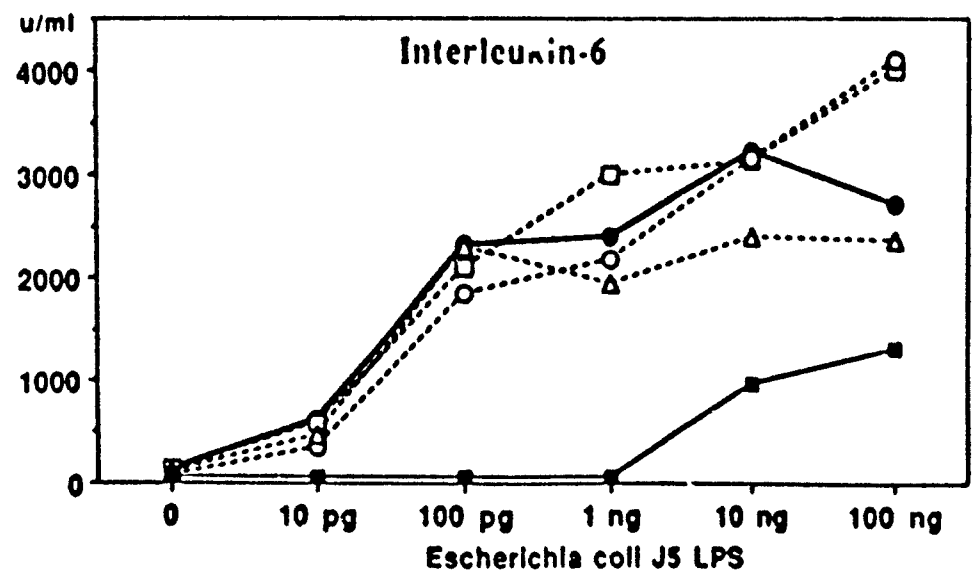
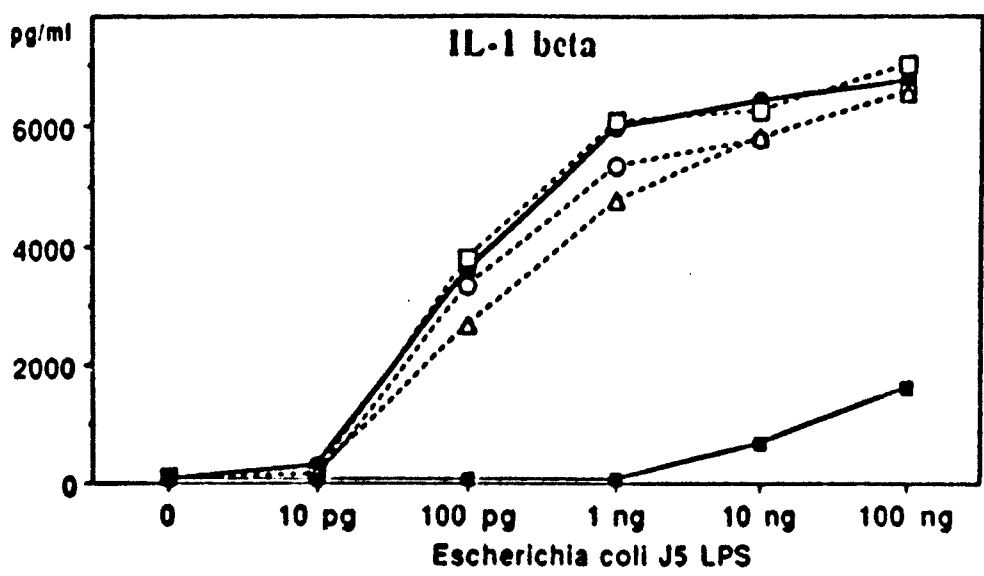
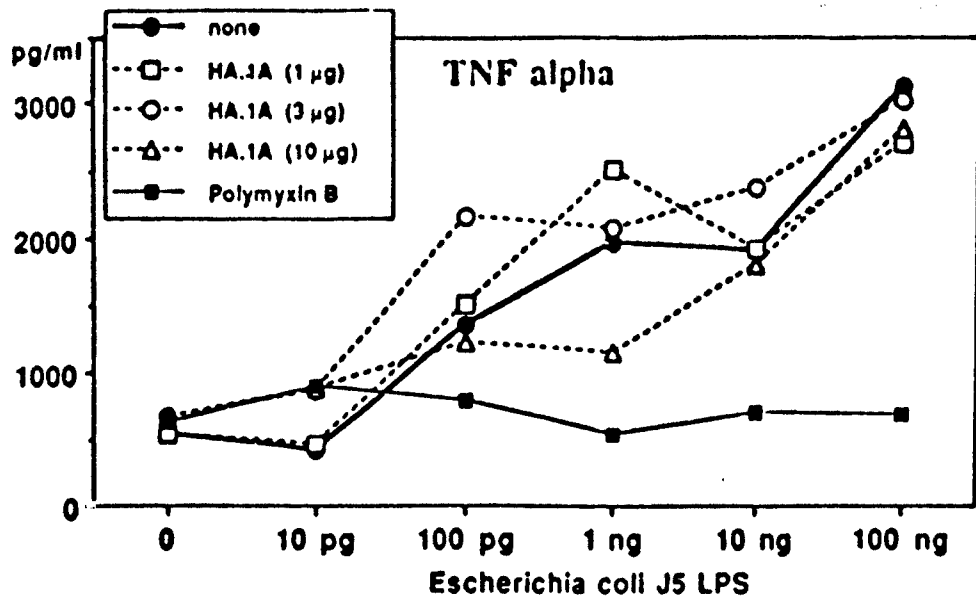
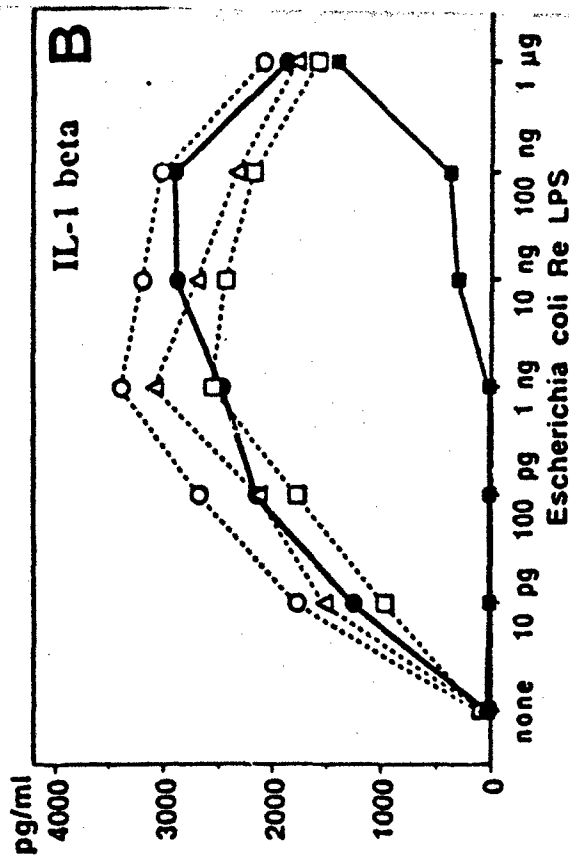
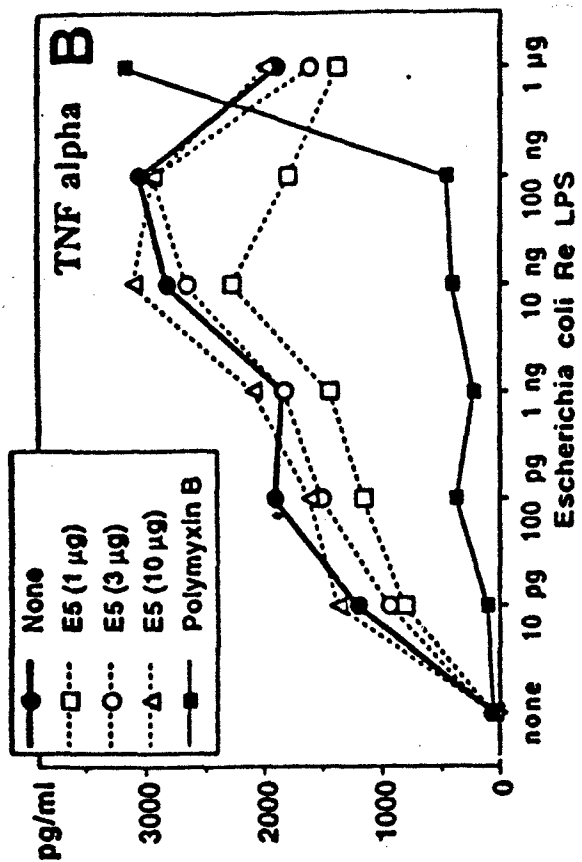
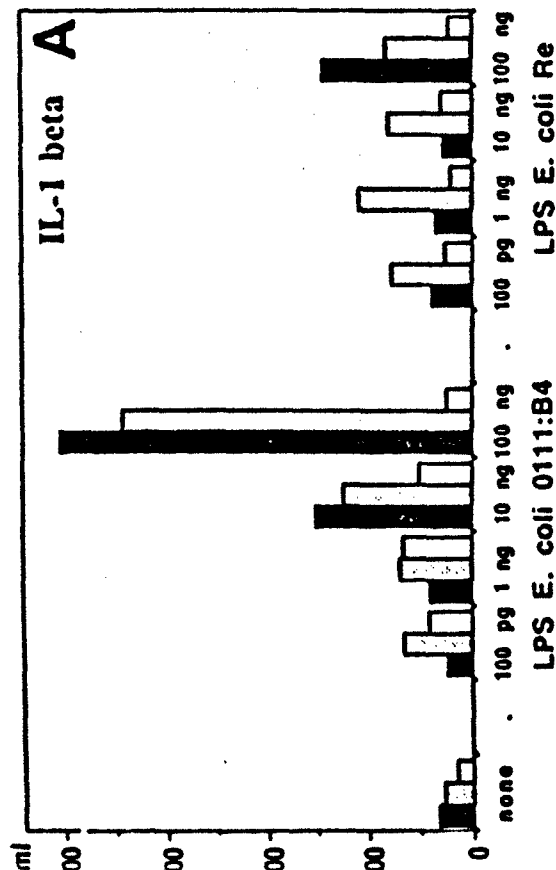
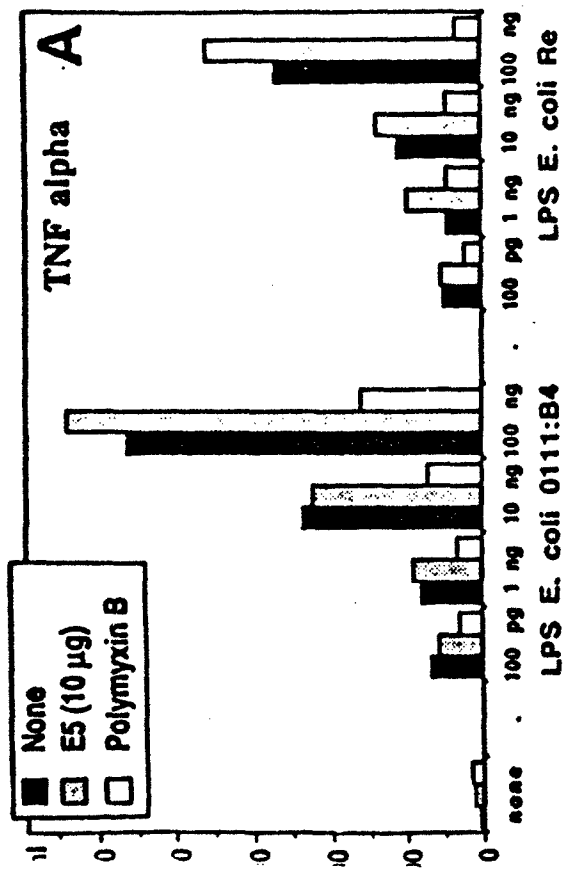


Fig 5





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PROTECTIVE EFFECTS OF ANTI-O POLYSACCHARIDE AND ANTI-LIPID A
MONOCLONAL ANTIBODIES ON THE OVINE PULMONARY CIRCULATION

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**Condensed title: PROTECTIVE EFFICACY OF MONOCLONAL ANTIBODIES
AGAINST ENDOTOXIN IN SHEEP**

KEY WORDS

antibody, monoclonal, lipopolysaccharide, endotoxin, thromboxane, sheep, pulmonary hypertension

ABSTRACT

Monoclonal antibodies directed to endotoxin can protect in some animal models against the pathophysiological effects of endotoxin infusion. When 0.02 $\mu\text{g/kg}$ of lipopolysaccharide (LPS) derived from E.coli O111:B4 was incubated in vitro for 2 hours with the murine IgG monoclonal antibody (MAb), 5B10, directed against the O-polysaccharide antigenic domain of E.coli O111:B4, and then the mixture was infused into sheep, we noted significant protection. The initial temperature peak was decreased ($p < 0.001$ vs LPS control), and both the second temperature peak and leukopenia were abolished. The pulmonary artery pressure (PAP) elevation was diminished (mean peak PAP 23.2 ± 2.5 mmHg, $p < 0.05$ vs LPS control) and the peak plasma thromboxane B_2 ($\text{Tx}B_2$) level was reduced (mean peak $\text{Tx}B_2$ level 0.50 ± 0.15 ng/ml, $p < 0.05$ vs LPS control). In contrast, preincubation of the LPS with a human IgM MAb, HA-1A, directed against the core glycolipid of the LPS molecule provided only a minor protective effect on core temperature increase, but no reduction of the increase of plasma $\text{Tx}B_2$ or PAP.

INTRODUCTION

Despite improvements of antibiotic and supportive therapy, gram-negative bacterial sepsis remains a common and lethal condition with a high mortality rate in critically ill patients (4,23). The complications of sepsis, including shock and the adult respiratory distress syndrome, produce death in over 50 percent of septic patients (9,15). lipopolysaccharide (LPS, endotoxin), a component of the Gram-negative bacteria cell wall, is believed to be causally related to the development of ARDS and the high mortality of the sepsis syndrome (6,28,34). Consequently strategies to treat or prevent Gram-negative sepsis have focused upon neutralizing this toxic molecule. The immunotherapy of endotoxemia has been studied for over 20 years. Anti-endotoxin antibodies have been examined for the neutralization of LPS with conflicting results. Although a recent multicenter clinical trial of an anti-lipid A human monoclonal antibody (MAb), HA-1A, in patients with Gram-negative sepsis and septic shock demonstrated a 39% decreased mortality in the treated group (36), other studies have not confirmed the protective effects afforded by antibodies directed to the core region of LPS (2,11,32). Antibodies directed to the core glycolipid will probably be used clinically for the treatment of Gram-negative sepsis patients, although the protective effects of individual antibodies remain controversial.

In order to evaluate the protective effects of the human monoclonal anti-lipid A antibody HA-1A, we compared the protective effects on pulmonary hemodynamics, mediator release, core temperature and circulating leukocyte concentration of HA-1A with a murine IgG

monoclonal antibody directed to the O polysaccharide of LPS (5B10) in awake sheep.

METHODS AND MEASUREMENTS

SHEEP PREPARATION

Twenty two healthy Suffolk sheep weighing 25-35 kg were studied. This study was approved by the Massachusetts General Hospital Subcommittee on Animal Care and Research. Sheep with evidence of an infection on any study day were excluded. The criteria for infection were described previously (33). No animals in this study had undergone prior experimentation. The sheep were studied awake in a Babraham veterinary cage with free access to food and water.

At the start of each experiment, after 2% xylocaine infiltration anesthesia, an 8-Fr. introducer (Cordis Co. Miami, FL) was inserted into the jugular vein. A sterile 7-Fr. flow-directed thermodilution catheter (American Edwards 93A-131H-7F, Santa Ana, CA) was advanced into the pulmonary artery through the introducer to monitor central pressures and core temperature.

Pulmonary hemodynamic values including the pulmonary artery pressure (PAP) and pulmonary capillary wedge pressure (PCWP) were measured with a calibrated pressure transducer (Hewlett-Packard 1280C, Palo Alto, CA) that was leveled to the front shoulder as a reference for the right atrium, and recorded continuously with a four-channel recorder (Hewlett-Packard 7754B, Palo Alto, CA). Cardiac output (CO) was determined as the mean of three measurements by thermodilution, injecting 5 ml of 0°C Ringer's lactate solution through the proximal port of the pulmonary artery catheter. CO was calculated by a cardiac output computer (American Edwards Laboratories, model 9520A, Irvine, CA). Blood samples and measurements of mean PAP, PCWP and CO were

taken at 15-minute intervals for the first 90 minutes and subsequently at 30-minute intervals. PAP was taken as an electrical mean and PCWP was measured at end expiration. The pulmonary vascular resistance (PVR) was calculated as $(\text{PAP}-\text{PCWP})/\text{CO}$.

Blood samples were withdrawn through the proximal port of the pulmonary artery catheter into plastic syringes. The samples were transferred into glass tubes containing 0.05 ml of a 15% solution of EDTA and 20 μg of indomethacin; 40 μl of blood was removed for a leukocyte count measured by a Coulter Counter (Model ZF, Coulter Electronics, Hialeah, FL). The remainder of the sample was immediately placed on ice and centrifuged (Damon/IEC, model PR-6000) at 1250g and 4°C for 10 minutes. The supernatant was pipetted into polypropylene tubes and stored at -20°C. The plasma thromboxane B_2 (TxB_2) level was determined by radioimmunoassay using an anti-thromboxane B_2 antibody with methods previously described (1,19).

ENDOTOXIN PREPARATION

Escherichia coli O111:B4 endotoxin (List Biologicals, Campbell, CA) was used for the experiments. The lyophilized LPS of a single batch (5 mg/vial) was reconstituted with sterile nonpyrogenic water and diluted to produce a stock solution of 20 $\mu\text{g}/\text{ml}$, which was stored at 4°C. At the beginning of each experiment, the stock solution was thoroughly vortexed and appropriately diluted for injection using sterile, endotoxin-free pipette tips (National Scientific Supply Company, Inc. San Rafael, CA), polystyrene tubes (Falcon, Becton Dickinson, Lincoln Park, NJ), and saline.

ANTIENDOTOXIN ANTIBODY

The monoclonal antiendotoxin antibodies used in these experiments were obtained from Centocor, Malvern, PA. The human monoclonal antibody (MAb), HA-1A, is an IgM antibody that binds to the lipid A domain of endotoxin and is produced by the stable heteromyeloma cell line A6(H4C5) (29). HA-1A was provided as a 5 mg/ml solution in 5% human serum albumin (HSA). The murine MAb, 5B10 belongs to the IgG2a subclass and specifically reacts in vitro with E.coli O111:B4 bacteria and its LPS antigen (7). This antibody was provided as a solution containing 1.75 mg protein/ml in a buffered solution. Both antibodies were stored at 4°C, and were immediately diluted with sterile saline and preincubated with LPS before each experiment.

EXPERIMENTAL PROTOCOL

Sheep were randomly divided into four groups. Six sheep were challenged with 0.02 µg/kg of E.coli O111:B4 LPS without any antibody. Six sheep received an intravenous infusion of an in vitro incubated mixture of 0.02 µg/kg E.coli O111:B4 LPS and 0.02 mg/kg HA-1A. Six sheep received an in vitro incubated mixture of 0.02 µg/kg E.coli O111:B4 and 2 mg/kg 5B10 intravenously. Four sheep received an infusion of 0.02 mg/kg of 5B10 without an LPS challenge. Since the MAb HA-1A was provided in 5% HSA, we added HSA to the 5B10 and LPS control incubated mixtures. Each mixture was incubated at 37°C for 2 hours in a water bath with continuous shaking. The mixture was vortexed every 15

to 20 minutes during incubation. Then the mixture was intravenously infused into the sheep over 5 minutes beginning at time zero.

STATISTICAL ANALYSIS

All data were stored in an IBM PC/AT computer. Mean values and standard errors of hematological, biochemical and physiological values were calculated and reported. Data were averaged over time and compared with the baseline by paired t-tests. Temperature, hematological, and hemodynamic data were statistically compared over time as well as between treatment groups using analysis of variance for repeated measures (SAS version 6, SAS Institute, Cary, NC). Plasma TxB_2 data were statistically analyzed after a logarithmic transformation. A value of $p < 0.05$ was considered to be significant.

RESULTS

The alterations of core temperature, leukocyte concentration, pulmonary artery pressure, and pulmonary vascular resistance before and up to 4 hours after challenge in all four groups are presented in figure 1. Plasma TxB_2 concentration is displayed for these four groups in figure 2. In order to confirm that there was no contamination of our solutions by LPS, HA1A and HSA were each infused alone into two sheep. There were no physiological or hematological changes observed (data not shown). The incubated mixture of MAb:5B10 and HSA was infused into four sheep (see figure 1 and 2)

LPS control group: After challenge there was an early core temperature elevation occurring from 45 to 75 minutes, reaching a first peak ($+1.30 \pm 0.20^\circ\text{C}$) at 60 minutes after starting infusion of the preincubated mixture of LPS and HSA (see figure 1). A delayed second temperature peak ($+1.34 \pm 0.25^\circ\text{C}$) was measured at 150 minutes. Both temperature elevations persisted from 45 to 240 minutes after challenge. A significant reduction of WBC (mean maximum reduction - $2,568 \pm 1025 \text{ WBC/mm}^3$) lasted from 60 to 240 minutes. The PAP was elevated from 15 to 45 minutes and at 120, 150 and 240 minutes. The PVR was increased between 30 and 60 minutes. The plasma TxB_2 concentration increased at 30 minutes and reached a mean peak level of $2.51 \pm 0.73 \text{ ng/ml}$ at 60 minutes (see figure 2).

LPS + 5B10: A single peak of elevated core temperature occurred from 75 to 90 minutes and the maximum mean temperature elevation ($+0.67 \pm 0.18^\circ\text{C}$) was significantly decreased ($p < 0.001$) as compared with the LPS control and the LPS + HA-1A groups. The second tempera-

ture elevation did not occur. Only a minor but significant leukocyte decrease was measured at 75 minutes, however a leukocytosis occurred from 150 to 240 minutes. The PAP and PVR increase were significantly attenuated. There was no significant increase of plasma TxB_2 .

LPS + HA-1A: After the infusion of this preincubated mixture, the core temperature was elevated from 45 to 75 minutes and reached the first peak ($+1.22 \pm 0.08^\circ\text{C}$) at 60 minutes, the delayed temperature elevation was decreased ($p < 0.05$) when compared with the LPS control group. A transient leukopenia was measured from 45 to 90 minutes, the value returning to the baseline level by 120 minutes. The PAP and PVR were increased from 30 to 60 minutes. The plasma TxB_2 level was increased at 30 minutes and there was no significant difference when compared with the LPS control group.

DISCUSSION

The major findings of this study are: 1) When the O-polysaccharide specific murine monoclonal antibody (5B10), was incubated in vitro for two hours with E. coli 0111:B4 LPS and then infused into awake sheep, it moderated the release of thromboxane and the subsequent pulmonary vasoconstriction and hypertension as well as the febrile response and leukopenia; 2) in-vitro incubation for 2 hours of the same dose of LPS with a human monoclonal anti-lipid A antibody (HA-1A) had only minor effects on the response to infusion of this mixture as measured by a decreased febrile response with a single temperature peak and a briefer leukopenia. However there was no reduction of the plasma TxB_2 increase, pulmonary vasoconstriction or pulmonary artery hypertension as compared to the control.

The pathophysiology of Gram-negative sepsis is incompletely understood. Clinical and experimental studies have demonstrated that infusion of LPS mimics the toxic effects of Gram-negative sepsis (5,14,22,25). Consequently, strategies to treat or prevent Gram-negative sepsis have focused upon neutralizing LPS. Anti-endotoxin antibodies have been studied for neutralization in various animal models with conflicting results. O-specific monoclonal antibodies protect animals from challenge with homologous but not heterologous bacterial strains (7,8,16), so their clinical use is limited. Because the core-lipid A region of LPS represents a common structure and antigen in many Gram-negative bacteria, antibodies against core-lipid A of the LPS molecule have been extensively studied in patients and animal models.

Since the cardiovascular system of sheep responds to endotoxin infusion in a similar fashion to humans and because low doses of endotoxin produce significant mediator, hematological and pulmonary hemodynamic responses in awake sheep (33), we chose this endotoxemia model for our study. Since the protective effects of antisera or antibodies to endotoxin may be overcome by large challenging LPS doses, only a small dose of LPS was infused in this preincubation study. Intravenous infusion of E. coli 0111:B4 LPS at 0.02 µg/kg body weight, reliably induces TxB_2 release and physiologically important acute pulmonary vasoconstriction and hypertension, as well as the systemic effects of a biphasic febrile response and leukopenia in awake sheep. This dose of LPS is on the steep portion of a dose-response curve for these parameters (33).

In vitro incubation of the anti-lipid A antibody (HA-1A) with LPS did not attenuate thromboxane release and the pulmonary hemodynamic responses to LPS infusion in awake sheep, but did inhibit the second peak of temperature elevation and reduce the duration of the leukopenia. We were unable to demonstrate protective effects of the anti-lipid A antibody (HA-1A) on the pulmonary circulation. It should be noted that incubation of LPS with the O-specific antibody (5B10) also did not totally blunt the pulmonary and systemic response to infusion of this mixture into awake sheep, although significant attenuation did occur.

To allow each antibody a sufficient time to neutralize the LPS, we chose the technique of incubating the antibody for two hours with LPS before infusing the mixture into sheep. During incubation we added a 1,000 fold excess of antibody to LPS by weight. Despite our attempts

to enhance antibody neutralization of LPS, we failed to demonstrate a significant protective effect of the anti-lipid A antibody on the pulmonary circulation. Several factors may be responsible for this failure of the MAb HA-1A to neutralize LPS. The complexity of the LPS molecule is one factor. Although it is widely believed that lipid A is the toxic portion of the LPS molecule (24), other studies reported that other endotoxin components may also contribute to the toxic reaction, perhaps by activating monocytes and macrophages releasing cytokines (12,17). It is thus possible that other components of LPS may also participate in producing the septic response. Alternatively, another possible explanation of our failure to protect the pulmonary circulation is related to the structure of smooth LPS. The O-side chain and core oligosaccharides of LPS may restrict the access of the antibody to epitopes in the core region of the LPS molecule in vitro. Gigliotti and Shenep reported that monoclonal antibodies directed to lipid A were unable to bind to intact smooth strains of *E. coli* (10). Nys et al. demonstrated that anticore antibodies can protect mice against lethal challenge with smooth LPS, but they did not observe any serologic reactivity of anticore antibodies with smooth LPS in vitro (21).

Dunn et al. studied the specific MAb 5B10 and another IgG anti-lipid A antibody and reported that the specific antibody produced greater protective effects than the anti-lipid A antibody. In that study a very large dose of anti-lipid A antibody was needed to produce the same protective effects as the specific antibody (8). In our study, the same 1,000 fold excess dose of the anti-lipid A antibody HA-1A did not prevent thromboxane release and pulmonary vasoconstrict-

tion due to infusion of this LPS mixture while an equal amount of the specific antibody 5B10 afforded considerable protection. Possibly, an even larger dose of anti-lipid A antibody would be needed to produce a protective effect on the pulmonary circulation.

Although some studies have demonstrated the cross-protective effects against LPS afforded by polyclonal antibodies or monoclonal antibody against the core region of LPS (18,20,27,35), other studies do not demonstrate cross-protection (2,11). Recently Wheeler et al. reported that pretreatment of sheep with a murine anti-lipid A IgM MAb (2mg/kg) only moderated the early PAP rise after subsequently intravenous injection of LPS:E.coli O55:B5 at a dose of 0.75 µg/kg (32). They also noted that incubating the MAb with 0.75 µg/kg of LPS for 30 minutes before injection in a weight ratio of 2,000 fold MAb excess, significantly blunted the increase of PAP; however, no other measured variable (lung mechanics, gas exchange, lung lymph flow rate and circulating WBC counts) was moderated by incubation with the MAb. In an in vitro limulus lysate assay, incubation of LPS with this MAb in molar ratio from 1:10³ to 1:10⁶ did not reduce LPS-induced limulus activation (32). These studies suggested that this anti-endotoxin MAb did not effectively neutralize the effects of E.coli endotoxin in the sheep.

Warren et al. studied the neutralization of LPS by rabbit antisera, and reported that neutralization of homologous LPS was only partly mediated by immunoglobulin. Antisera to rough mutant bacteria neutralized heterologous LPS slowly and in a manner unrelated to the concentration of antibody (31). Siber et al. were unable to detect cross reactive antibodies in polyclonal antisera to rough mutants, and

suggested that the protective effects of antisera to mutant bacteria may be in part related to a polyclonal antibody response (26). Their results were recently confirmed by Heumann et al. and Baumgartner et al. using human antisera (3,13).

It is unclear why the specific antibody 5B10 did not completely blunt the response to LPS in our study. The sheep has abundant pulmonary intravascular macrophages and is one of the most sensitive animals to LPS (30). Perhaps tiny amounts of free LPS or complexes of LPS with MAb can activate these macrophages and other inflammatory cells to release mediators, leading to lung injury and systemic responses to endotoxemia.

In our study, we compared the protective effects of a specific MAb and an anti-lipid A MAb on the pulmonary circulation of awake sheep by infusing of an in vitro incubated mixture of antibody with E.coli O111:B4 LPS. We failed to demonstrate a pulmonary protective effect of the anti-lipid A antibody HA-1A. In contrast, incubation with the specific antibody 5B10, directed against the O-side chain of E.coli O111:B4 LPS markedly attenuated the increase of plasma thromboxane B₂ as well as the pulmonary vasoconstriction and hypertension and inhibited the febrile response of awake sheep. Although several potential reasons may explain the failure of the anti-lipid A antibody to protect the sheep against LPS, we believe more basic research is required to learn the precise nature of the protective effects of anti-lipid A antibody.

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LEGEND

FIGURE 1. Changes in core temperature, leukocyte count, pulmonary artery pressure and pulmonary vascular resistance after infusion of in vitro preincubated mixtures of LPS + HSA (n=6, —●—), LPS + HA1A + HSA (n=6, --O--), LPS + 5B10 + HSA (n=6, — —), 5B10 + HSA (n=4, ..▲..). Values are mean $\pm$ sem, * $p < 0.05$, ** $p < 0.001$ compared with LPS + HSA control group over time.

FIGURE 2. Plasma thromboxane level after intravenous infusion of mixture of incubation of LPS + HSA (n=6, —●—), LPS + HA1A + HSA (n=6, --O--), LPS + 5B10 + HSA (n=6, — —), 5B10 + HSA (n=4, ..▲..). Values are mean $\pm$ sem, * $p < 0.05$, ** $p < 0.001$ compared with LPS + HSA control group over time.

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Binding and Neutralization of Endotoxin by *Limulus* Antilipopolysaccharide Factor

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In order to examine the ability of *Limulus* antilipopolysaccharide factor (LALF) to bind lipopolysaccharide (LPS), we purified LALF to homogeneity from *Limulus* amoebocyte lysate and coupled it covalently to agarose beads. LALF-coupled beads captured more tritiated LPS from rough and smooth strains of gram-negative bacteria than did control human serum albumin-coupled beads. Unlabeled homologous and heterologous LPS competed for the binding of ³H-LPS to LALF-coupled beads. LALF bound LPS in a dose-dependent manner as assessed by the precipitation of LPS-LALF complexes with 50% saturated ammonium sulfate. We also studied the ability of LALF to neutralize LPS. LPS preincubated with LALF was less mitogenic for murine splenocytes, was less pyrogenic in the rabbit fever assay, was less lethal in mice which had been sensitized to LPS with actinomycin D, and induced less fever, neutropenia, and pulmonary hypertension when infused into sheep. Our findings extend prior studies which suggested that LALF binds to and neutralizes LPS from multiple strains of gram-negative bacteria.

Despite the availability of antibiotics capable of rapidly killing most gram-negative bacteria, infections caused by these organisms, with the subsequent development of sepsis, shock, and multisystem organ failure, continue to be a major clinical problem. Bacterial lipopolysaccharide (LPS) is felt to play an important role in the pathophysiology of severe gram-negative infections. Accordingly, a strategy for the treatment of gram-negative sepsis has been to attempt to develop a means to neutralize or clear LPS from the bloodstream before the induction of irreversible pathology.

One such approach has been to passively infuse immunoglobulin directed at LPS. Polyclonal (15, 17, 41) and monoclonal (7, 10, 18, 21) antibodies directed at the O polysaccharide of LPS protect against challenge with LPS in animal models. However, immunoglobulin directed against O polysaccharide is only protective against homologous strains of bacteria, making it difficult to utilize clinically. Polyclonal (4, 6, 17, 20, 48, 50) and monoclonal (14, 49) antibodies directed at the common core glycolipid structure on LPS have also been reported to give protection in animal models and clinical trials. These immunoglobulin preparations are reported to protect against most gram-negative strains, although the protection in animal models is less than that of antibody directed at the O polysaccharide.

There are relatively few proteins which have been reported to bind and neutralize LPS. Two such proteins are

polymyxin B and bactericidal/permeability-increasing protein (19, 45). Polymyxin B has been known for over two decades to protect animals against challenge with LPS (8, 31, 34-36) and against gram-negative infection (3, 9), but it is believed to be too toxic for routine clinical use. Bactericidal/permeability-increasing protein was recently reported to inhibit LPS-induced stimulation of neutrophils and coagulation of *Limulus* amoebocyte lysate (LAL) (19). Further studies will be needed to assess whether it will be helpful as a therapeutic agent for endotoxemia.

In 1982, an anticoagulant which inhibited the endotoxin-mediated activation of the *Limulus* coagulation system was identified in the amoebocytes of the hemolymph of the horseshoe crabs *Tachyplesus tridentatus* and *Limulus polyphemus* (40). This factor has since been isolated and characterized (22), and it is a single-chain polypeptide with a molecular weight of 11,600 (26). The primary structure of the molecule consists of 102 amino acids and is partially homologous with structures of molecules in the lactalbumin-lysozyme family (1).

Several experiments suggest that this factor acts by binding to LPS. First, the factor inhibits activation of LAL by LPS, but not by another activator, (1-3)- β -D-glucan (40). Second, the factor lyses erythrocytes that are sensitized by prior incubation with LPS but does not lyse unsensitized cells, and the ability to hemolyze is inhibited by excess LPS added to the reaction mixture (29). Third, the activation of cultured human endothelial cells by LPS is decreased in a dose-dependent manner if the LPS is preincubated with the factor (11). Fourth, the factor forms precipitation lines when reacted with LPS in agarose double-diffusion gels (27). Because of these studies, the factor has been called *Limulus* anti-LPS factor (LALF) (1, 22).

A preliminary study suggested that the lethal toxicity of LPS in rats was diminished by preincubation with LALF (43). Recently, LALF has also been shown to attenuate the

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toxic effects of meningococcal lipooligosaccharide in rabbits (2). To test the hypothesis that this factor has antitendotoxic properties in other systems, we purified LALF to homogeneity from LAL and examined its ability to bind to endotoxin from several different gram-negative bacteria by measuring the amount of ^3H -LPS that the factor captured when covalently coupled to agarose beads and by precipitating ^3H -LPS-LALF complexes with ammonium sulfate. We then assessed the ability of LALF to decrease the mitogenic activity of LPS on murine splenocytes in vitro and to decrease LPS-induced pyrogenicity in rabbits, lethality in mice treated simultaneously with actinomycin D, and fever, leukopenia, and pulmonary artery vasoconstriction and hypertension in awake sheep. Our results confirm and extend prior studies and indicate that LALF binds to and neutralizes LPS from numerous different gram-negative strains both in vitro and in vivo.

MATERIALS AND METHODS

LPSs. Unlabeled LPS from *Salmonella typhimurium*, *Klebsiella pneumoniae*, and *Serratia marcescens* were purchased from List Co. (Campbell, Calif.). Unlabeled LPS from *Escherichia coli* O113 was prepared by the hot phenol method as described by Rudbach et al. (39).

Cultures of *S. typhimurium* G30, *E. coli* O111:B4, *E. coli* O18, and *E. coli* J5 were the kind gifts of Paul Rick (Uniformed Health Services University and Health Sciences, Bethesda, Md.), David Morrison (University of Kansas Medical Center, Kansas City), George Siber (Dana Farber Cancer Institute, Boston, Mass.), and Jerald Sadoff (Walter Reed Army Institute of Research, Washington, D.C.), respectively. Cultures of *E. coli* O4, O6, O16, O25, and O75 were the kind gifts of Alan Cross (Walter Reed Army Institute of Research).

Biosynthetically radiolabeled isolates of all of the "smooth" *E. coli* strains were prepared by growing the organisms in the presence of ^3H -acetate and then subjecting them to hot phenol extraction (44). Briefly, we grew cultures of each organism to an optical density of 0.9 at 540 nm (with a path length of 1.0 cm) in broth containing (per liter) 22.5 g of yeast extract, 11 g of peptone, 4 g of monobasic potassium phosphate, 16.8 g of dibasic potassium phosphate, and 10 g of glucose, with 10 mCi of ^3H -acetate per 100 ml of broth. The cells were chilled and washed three times in saline, and the LPS was extracted by the hot phenol method (46). The preparations were then treated first with DNase and RNase and then with pronase (Sigma Chemical Co., St. Louis, Mo.) according to the method of Romeo et al. (38). The concentrations of LPS were estimated by a spectrophotometric LAL gelatin assay utilizing an *E. coli* O113 LPS standard containing 10 endotoxin units per ng (lot 20; Associates of Cape Cod, Falmouth, Mass.). These results were similar to those obtained by weight. Solutions of ^3H -LPS were adjusted to 1 μg of LPS per ml (as *Limulus* biological activity), and counts per minute per microgram were calculated by counting a 0.4-ml volume combined with 4.5 ml of Optiflor scintillation fluid (Packard, Downers Grove, Ill.). The different LPSs contained the following counts per minute per microgram: *E. coli* O4, 10,490; *E. coli* O6, 7,200; *E. coli* O18, 6,150; *E. coli* O16, 17,100; *E. coli* O25, 11,040; *E. coli* O75, 4,700; *E. coli* O111:B4, 4,040. More than 99% of each radiolabeled LPS was demonstrated to remain in the water phase after a 1:1 ether-water extraction at pH 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of each LPS resulted in a regularly spaced band

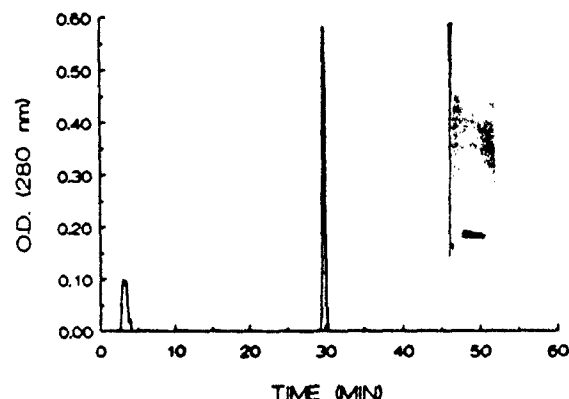


FIG. 1. Purity of LALF as assessed by high-performance liquid chromatography and SDS-PAGE. Conditions for SDS-PAGE are given in Materials and Methods. The apparent molecular weight of the protein band shown was estimated to be 15,000 by comparing it with molecular weight markers (not shown). O.D., optical density.

pattern typical of LPS when stained with silver. Similar regularly spaced band patterns were obtained when the gels were analyzed by autoradiography.

Radiolabeled LPS from *E. coli* J5 was made by growing the organisms in broth containing ^3H -acetate as described above and extracting the LPS as described by Galanos et al. (13). This LPS contained 22,000 cpm/ μg .

Radiolabeled LPS from *S. typhimurium* G30 was prepared by growing the organisms in PPBE broth (per liter: 10 g of peptone, 1 g of beef extract, and 5 g of NaCl in 0.05 mM unlabeled D-galactose) containing 500 μCi of D-[1- ^3H]galactose per 100 ml of broth as described by Munford and Hall (23). This strain produces complete LPS only in the presence of galactose and incorporates exogenous galactose almost entirely into the LPS (30), thus ensuring that all of the extracted label is found in LPS. The LPS was then extracted and standardized as described above. This LPS contained 16,800 cpm/ μg .

Purification of LALF from *L. polyphemus* amoebocytes. LALF was purified from LAL as previously described (43) by using a spectrophotometric LAL assay to monitor inhibition of LPS-induced lysate activation (28). Briefly, amoebocytes from *L. polyphemus* were collected under endotoxin-free conditions, lysed by the addition of distilled water, and centrifuged at $5,000 \times g$ for 30 min. The pellet was extracted with 3 M urea. The extract was filtered through a membrane with a 30,000-Da cutoff and concentrated by a membrane with a 8,000-Da cutoff. The retentate was applied to a cation exchange column (CM Sepharose) equilibrated with 3 M urea-10 mM ammonium acetate (pH 5.5) and step eluted with NaCl at 0.15, 0.25, 0.5 M. The 0.5 M NaCl peak was directly applied to a C-4 reversed-phase column (Vydac, Hesperia, Calif.) equilibrated with water-0.2% trifluoroacetic acid. The column was step eluted with 25, 35, and 50% isopropanol containing 0.2% trifluoroacetic acid. The 50% isopropanol peak was lyophilized and reconstituted immediately before use. The final product was estimated to be >95% pure by reversed-phase high-performance liquid chromatography and SDS-PAGE (Fig. 1). SDS-PAGE was carried out with a Pharmacia PhastSystem at 8 to 25% acrylamide with 2% cross-linking and the manufacturer's Tris acetate buffer system. Staining was performed with Coomassie

R 350. The amino acid sequence of LALF was determined up to residue 30 and was found to be essentially identical to that reported by Muta et al. (26).

Binding assays. The ability of LALF to bind LPS was assessed by two methods. In the first method, we measured the amount of LPS that was captured by agarose beads to which LALF had been covalently coupled. Control beads were coupled with human serum albumin (HSA).

LALF and HSA were covalently coupled to carbonyldiimidazole-activated agarose gel beads (Reacti-gel; Pierce, Rockford, Ill.) according to the manufacturer's directions. Briefly, the resin was washed and equilibrated in 0.1 M borate-0.9% NaCl, pH 8.5. Ten milligrams of protein was added per ml of packed bead volume, and the mixture was mixed end-over-end for 20 h at 4°C. The coupled gel was blocked with 2 M Tris buffer (pH 8.0) for 4 h and washed with pyrogen-free distilled water. By measuring protein concentrations after coupling, 8.5 mg of LALF and 9.2 mg of HSA were coupled per ml of gel. Binding assays were performed by combining triplicate samples of 1.0- μ g/ml radiolabeled LPS with a 2% (vol/vol) solution of LALF- or HSA-linked beads in a total of 0.4 ml of phosphate-buffered saline (PBS) (pH 7.0) in 1.5-ml polypropylene conical tubes. After 120 min of end-over-end mixing at 37°C, the tubes were centrifuged for 15 min at 10,000 $\times$ g. The supernatants were manually aspirated and saved and the pellets were washed twice with PBS. Counts per minute in the supernatants and pellets were measured in a liquid scintillation counter, adjusting for quenching by the internal standard method. The percentage of LPS bound to the beads was determined by dividing the total counts per minute in the pellet by the total counts per minute recovered and multiplying by 100. The total counts per minute recovered varied slightly depending on the type and quantity of LPS added and whether LALF- or HSA-coupled beads were used. Typically, 60 to 80% of added counts were recovered. Counts per minute not recovered with the beads or supernatant were quantitatively accounted for and shown to be bound to the tube walls by cutting the polypropylene tubes into quarters with a razor and counting in a similar manner. Competition binding experiments were performed by adding unlabeled homologous or heterologous LPS to reaction mixtures containing LALF-coupled beads and 1.0 μ g of ^{3} H-LPS per ml. Results are expressed as means $\pm$ standard deviations.

In the second method, ^{3} H-LPS was incubated in dilutions of LALF at 37°C. Complexes of ^{3} H-LPS bound to LALF were then separated from free ^{3} H-LPS by precipitation in half-saturated ammonium sulfate according to the method of Farr (12).

Specifically, 6,000 cpm of each smooth LPS tested was incubated in 150 μ l of twofold dilutions of LALF in 0.02 M PBS for 120 min at 37°C in a 1.5-ml Eppendorf microfuge tube. This corresponds to the following LPS concentrations in the final protein mixture: *E. coli* O4, 3.8 μ g/ml; *E. coli* O6, 5.6 μ g/ml; *E. coli* O16, 2.3 μ g/ml; *E. coli* O18, 6.5 μ g/ml; *E. coli* O25, 3.6 μ g/ml; *E. coli* O75, 8.5 μ g/ml. We elected to fix the number of counts per minute added rather than the concentration of LPS added in order to study the minimal concentration of each LPS that could be reliably detected. Following incubation at 37°C, the solution was cooled on ice for 15 min, an equal volume of iced saturated ammonium sulfate was added dropwise, and the solution was allowed to sit at 4°C for another 15 min. The tubes were then centrifuged at 12,000 $\times$ g for 15 min. Supernatants were carefully aspirated. Pellets were washed twice with 50% ammonium sulfate and resuspended in 300 μ l of PBS. The

quantity of LPS in the supernatants and pellets was assessed by counting 0.4 ml of a 1:7 dilution of each combined with 4.5 ml of Optiflor scintillation fluid (Packard). Quenching was minimal and was corrected for by the internal standard method. Less than 5% of each smooth LPS tested precipitated in PBS alone. However, more than 90% of the ^{3} H-LPS from rough mutant *E. coli* J5 precipitated in PBS-HSA alone, so that we were unable to test this LPS in the system. For most of the assays, recovery of added counts per minute was greater than 85% by this assay. At low protein concentrations of LALF or HSA (less than 50 μ g/ml), recovery was sometimes less, which we attributed to LPS binding to the walls of the tube. All assays were performed in duplicate, and the results are given as means. Results are expressed as the percentage of recovered counts per minute as calculated by the following formula: (counts per minute in the pellet/counts per minute recovered) $\times$ 100.

Mitogenic assay. The mitogenic assay was performed essentially as described by Jacobs and Morrison (16). Briefly, dilutions of LPS were preincubated in the presence of 10 μ g of LALF per ml for 2 h at 37°C. These mixtures were then incubated for 48 h in RPMI medium containing 5×10^6 spleen cells from CD-1 mice. One microcurie of ^{3} H-thymidine was next added to each well, and cells were incubated for an additional 16 h. Incorporated radioactivity was measured by using a mash harvester to disrupt the cells followed by scintillation counting. Each assay was done in quadruplicate, and the results are reported as means $\pm$ standard deviations. Staining of splenocytes with crystal violet following culture in the presence of 10 μ g of LALF per ml suggested that this concentration of LALF did not kill splenocytes. Concanavalin A was normally mitogenic in the presence of 10 μ g of LALF per ml.

Rabbit pyrogen assay. The rabbit pyrogen assay has been previously described (37). Male New Zealand White rabbits (2.5 to 3.5 kg) were used throughout the study. Glassware, needles, syringes, and PBS were pyrogen free. Rectal temperature was recorded every 3 min for 5 h after intravenous injection by using Thermistor probes connected to a telethermometer interfaced to an HP85 computer (Hewlett-Packard Co., Palo Alto, Calif.). Changes in temperature are expressed as maximum deviation from the base line recorded at the time of injection.

Earlier studies indicated that a dose of the same lot of *E. coli* O113 LPS at 150 ng/kg of body weight was on a sensitive portion of the dose-response curve (44). Accordingly, we preincubated LPS from *E. coli* O113 with a 100-fold excess of LALF by weight or in saline alone for 2 h at 37°C and administered these mixtures at an LPS dose of 150 ng/kg to groups of four acclimatized rabbits while recording their rectal temperature.

Actinomycin D mouse model. Mice are relatively resistant to the lethal effects of LPS injection. We therefore utilized actinomycin D as described by Brown and Morrison (5) and Pieroni et al. (32) to sensitize mice to submicrogram amounts of LPS. Dilutions of LPS were preincubated in saline alone or in 2.0 μ g of LALF per ml in saline at 37°C for 60 min and then combined 1:1 with 250 μ g of actinomycin D per ml immediately prior to injection. A total volume of 0.2 ml of this solution containing actinomycin D (25 μ g), LPS, and either saline or 200 ng of LALF was injected intraperitoneally into groups of six to nine CD-1 mice. Results are expressed as survivors per total mice at 72 h. The 50% lethal dose was calculated by the method of Reed and Muench (33). Statistics were calculated by logistic regression using

TABLE 1. Percentage of radiolabeled LPS captured by HSA or LALF coupled to agarose beads

³ H-LPS	% of LPS captured by:	
	HSA-coupled beads	LALF-coupled beads
<i>S. typhimurium</i>	26.0 ± 1.6	60.6 ± 2.3
<i>E. coli</i> O111:B4	13.7 ± 1.2	49.3 ± 1.5
<i>E. coli</i> O118	31.6 ± 1.0	89.9 ± 1.2
<i>E. coli</i> J5	59.5 ± 10.1	97.3 ± 0.2

the SAS statistical system (PC SAS version 6; SAS Institute, Cary, N.C.).

LPS-induced fever, neutropenia, and pulmonary hypertension in sheep. This model has been previously described (47) and represents an established model for measuring LPS effects upon pulmonary hemodynamics. The injection of nanogram-per-kilogram quantities of LPS induces reliable changes of core temperature, transient neutropenia, and mediator release with vasoconstriction and pulmonary artery hypertension. For this assay, LPS from *S. marcescens* was utilized because detailed dose response information was known from prior experiments (47).

Briefly, LPS was preincubated with a 200-fold excess of LALF or with pyrogen-free saline for 2 h at 37°C. Immediately following the incubation, 20 ng of LPS per kg (4 µg of LALF per kg) was intravenously injected into awake sheep. A sterile Swan Ganz catheter had been placed in the pulmonary artery via an introducer sheath in the external jugular vein. This catheter allowed us to measure and record pulmonary artery pressure and pulmonary artery core temperature continuously. We intermittently measured pulmonary artery occlusion pressure and injected 5 ml of sterile 0°C saline to measure cardiac output. Pulmonary vascular resistance was calculated by the standard formula (pulmonary artery pressure - pulmonary artery occlusion pressure)/cardiac output. Samples were obtained at intervals for quantitation of leukocytes by using a Coulter Counter.

All data for the sheep experiments were stored on a DEC LSI-11/73 computer and transferred to an IBM PC-286 computer for statistical analysis using the SAS statistical system. Core temperature, pulmonary hemodynamic, and hematologic data were statistically compared over time as well as between treatment groups by using analysis of variance for repeated measures. When, within a treatment group, effects were transient and occurred at different times, data were averaged over time and compared with those of the control population by *t* tests. For single comparisons, *P* < 0.05 was considered to be significant. All data are presented as means ± standard errors.

RESULTS

Ability of LALF- or HSA-coupled beads to capture radiolabeled LPS. LALF-coupled beads bound two to four times as much tritiated LPS from the smooth gram-negative strains studied than did the control HSA-coupled beads (Table 1). The LALF-coupled beads bound more than 95% of tritiated LPS from rough mutant *E. coli* J5, although nonspecific binding to the HSA-coupled beads was higher for this strain than for LPS from the smooth strains. Competition experiments were performed by adding unlabeled LPS to the reaction mixture containing LALF-coupled beads and ³H-LPS (1.0 µg/ml) from *S. typhimurium*. The addition of both

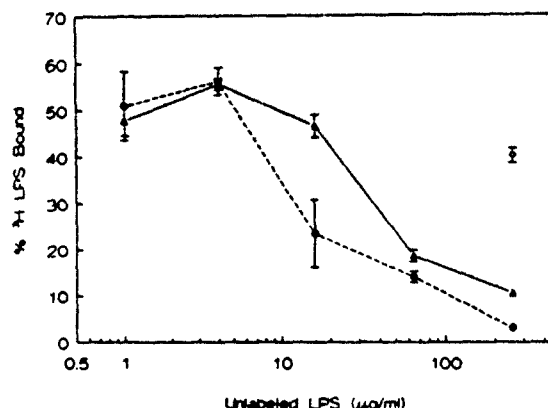


FIG. 2. Competition for binding of tritiated LPS to LALF-coupled beads by homologous and heterologous unlabeled LPS. Tritiated LPS (1 µg/ml) from *S. typhimurium* was added to the reaction mixture together with various concentrations of unlabeled LPS from *S. typhimurium* (●) or *K. pneumoniae* (Δ). The open diamond to the right of the figure represents the percentage of ³H-LPS bound to LALF-coupled beads in the presence of 1.2 mg of albumin per ml.

homologous and heterologous unlabeled LPS decreased the percentage of radiolabeled LPS bound to the beads (Fig. 2).

Ability of LALF to bind LPS as assessed by precipitation with saturated ammonium sulfate. LALF bound to ³H-LPS from six smooth *E. coli* strains in a dose-dependent manner (Fig. 3). Fifty percent of the recovered LPS was bound at LALF concentrations ranging from 250 to 700 µg/ml. When corrected for LPS concentration, this corresponds to the following ratios of LALF to LPS needed for half-maximal precipitation of each LPS: *E. coli* O4, 137:1; *E. coli* O6, 118:1; *E. coli* O16, 226:1; *E. coli* O18, 52:1; *E. coli* O25, 72:1; *E. coli* O75, 65:1.

Effect of LALF on mitogenic activity of LPS. *E. coli* O113 LPS which had been preincubated with LALF was less mitogenic than the saline control (Fig. 4). Similar findings were obtained when *S. typhimurium* LPS was used (data not shown).

Effect of LALF on LPS-induced fever in rabbits. *E. coli* O113 LPS which had been preincubated with LALF induced significantly less fever in rabbits than did LPS incubated in saline (*P* < 0.001) (Fig. 5).

Protective effect of LALF on actinomycin D-sensitized mice. The effect of LALF on mice sensitized with actinomycin D is shown in Table 2. As noted in Materials and Methods, mice received 25 µg of actinomycin D, dilutions of LPS, and either saline or 200 ng of LALF. Three experiments were performed, each with a range of LPS dilutions. In each experiment, more mice survived at critical LPS doses. The calculated 50% lethal dose obtained by using all mice was 35.0 ng per mouse for LPS in the presence of LALF and 2.8 ng per mouse in the presence of saline alone. By logistic regression with additive effects on the logit scale for log LPS dose and administration of LALF, both effects were highly significant (*P* < 0.001). The odds ratio for the probability of survival with the administration of LALF was 1.82.

Effect of LALF on LPS-induced fever, neutropenia, and pulmonary hypertension in sheep. Preincubation with LALF resulted in significantly less LPS-induced fever, neutropenia, pulmonary vasoconstriction, and hypertension than was

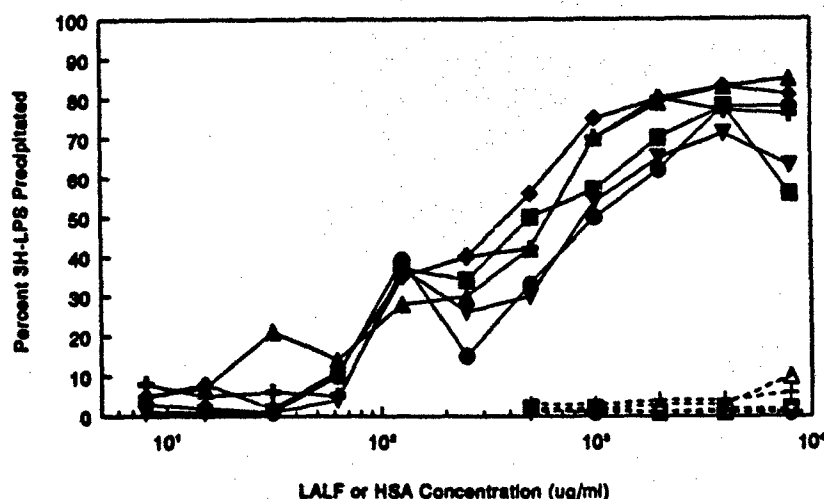


FIG. 3. Percentage of ^3H -LPS precipitated by different concentrations of LALF (solid figures, solid lines) or HSA (open figures, dashed lines), in 50% saturated ammonium sulfate as described in Materials and Methods. Symbols and denoted strains of *E. coli* are as follows: O4, plus signs; O6, circles; O16, triangles; O18, squares; O25, diamonds; O75, inverted triangles.

seen with control sheep (Fig. 6). The febrile response to LPS which had been preincubated with LALF was monophasic rather than biphasic, and the peak fever was lower and delayed ($P < 0.001$). Similarly, the increase in pulmonary artery pressure induced by LPS was markedly diminished and delayed by preincubation with LALF ($P < 0.05$). Preincubation with LALF abolished LPS-induced neutropenia ($P < 0.001$).

DISCUSSION

The major finding of our experiments is that LALF binds to LPS derived from several different gram-negative bacterial strains in vitro and that it can neutralize the toxic effects of LPS in in vitro and in vivo assays of biological activity. Our results extend prior studies using LALF in several ways. First, we have confirmed by direct binding assays that LALF binds to LPS from multiple strains of gram-negative bacteria. Second, we have shown that preincubation with

LALF diminishes the mitogenic response of LPS for murine splenocytes. Third, we have shown that LALF decreases the bioactivity of LPS in mice (which have been sensitized with actinomycin D), rabbits, and sheep. Each of these animal models represents an established assay for in vivo endotoxin activity.

There are a limited number of well-defined proteins which bind LPS and are capable of these antitoxic properties. Other substances reported to bind and neutralize the effects of LPS in biological assays and/or animal models include polymyxin B (3, 8, 9, 31, 34-36), bactericidal/permeability-increasing protein (19, 45), and immunoglobulin directed at the endotoxin core (4, 6, 14, 17, 20, 48-50). LPS also binds to plasma lipoproteins in the presence of disaggregating agents, and the complexes which are formed are less active than unbound LPS in numerous assays (24, 25, 42).

The results of the mitogenic assays, the mouse lethality test, and studies using LAL (data not shown) suggest that

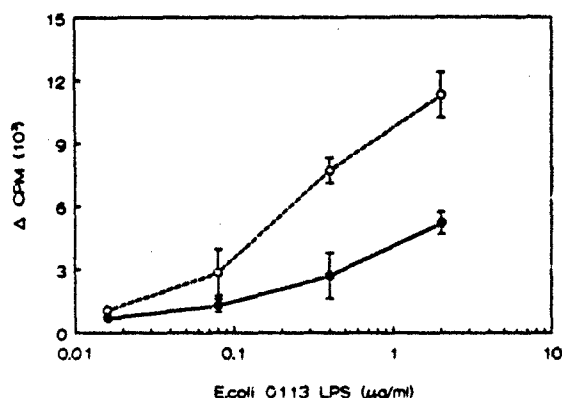


FIG. 4. Mitogenic response of *E. coli* O113 LPS which had been preincubated with LALF (10 $\mu\text{g}/\text{ml}$) (●) or saline alone (○).

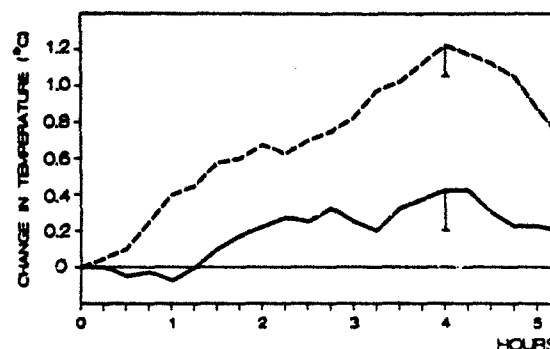


FIG. 5. Pyrogenic response in groups of four rabbits to intravenous injection of *E. coli* O113 LPS (150 ng/kg) which had been preincubated with a 100-fold excess of LALF (—) or saline alone (---). The maximal temperature of rabbits which were injected with LPS preincubated with LALF was significantly less than that of controls ($P < 0.001$).

TABLE 2. Protective effect of LALF in mice sensitized with antinomycin D

Dose	No. of surviving mice/total mice							
	Expt 1		Expt 2		Expt 3		Total*	
	Saline	LALF	Saline	LALF	Saline	LALF	Saline	LALF
LPS/mouse (ng)								
1,000.0	0/6	1/6	0/6	3/6	0/9	0/9	0/21	4/21
100.0	3/6	0/6	1/6	2/6	0/9	0/9	4/21	2/21
10.0	4/6	5/6	1/6	5/6	1/9	9/9	6/21	19/21
1.0	4/6	6/6	2/6	6/6	8/9	9/9	14/21	21/21
0.1	6/6	5/6	2/6	5/6	9/9	9/9	17/21	19/21
Saline alone	6/6	6/6	6/6	6/6	9/9	9/9	21/21	21/21

* Fifty-percent lethal doses were 2.8 ng of LPS with saline and 35.0 ng of LPS with LALF.

approximately a 10-fold excess of LALF by weight is needed for neutralization, a relationship which has been previously shown for the inhibition of endothelial cell activation (11). Estimation of the molar ratios needed for effective neutralization is not possible owing to uncertainty as to the effective molecular weight of the micellar form of LPS in aqueous solution.

Direct comparison with polymyxin B is difficult owing to the use of different model systems. Polymyxin B has been reported to neutralize LPS when premixed in a 115:1 ratio by weight in a chick embryo lethality assay (36), in a 300:1 ratio

in a murine lethality assay in which the mice were sensitized by adrenalectomy (34), and in a 50:1 ratio in assays using the Schwartzman reaction and neutropenia in rabbits (8, 35) and hypotension in dogs (31). We used 100:1 and 200:1 ratios of LALF to LPS by weight in rabbits and sheep, respectively. Using these doses, there was a substantial decrease in the effect of LPS challenge in each assay. The neutralization in sheep was particularly pronounced in that LPS-induced pulmonary artery hypertension was almost eliminated and neutropenia was not seen. Our results are in agreement with the findings of Alpert et al., who recently reported that a

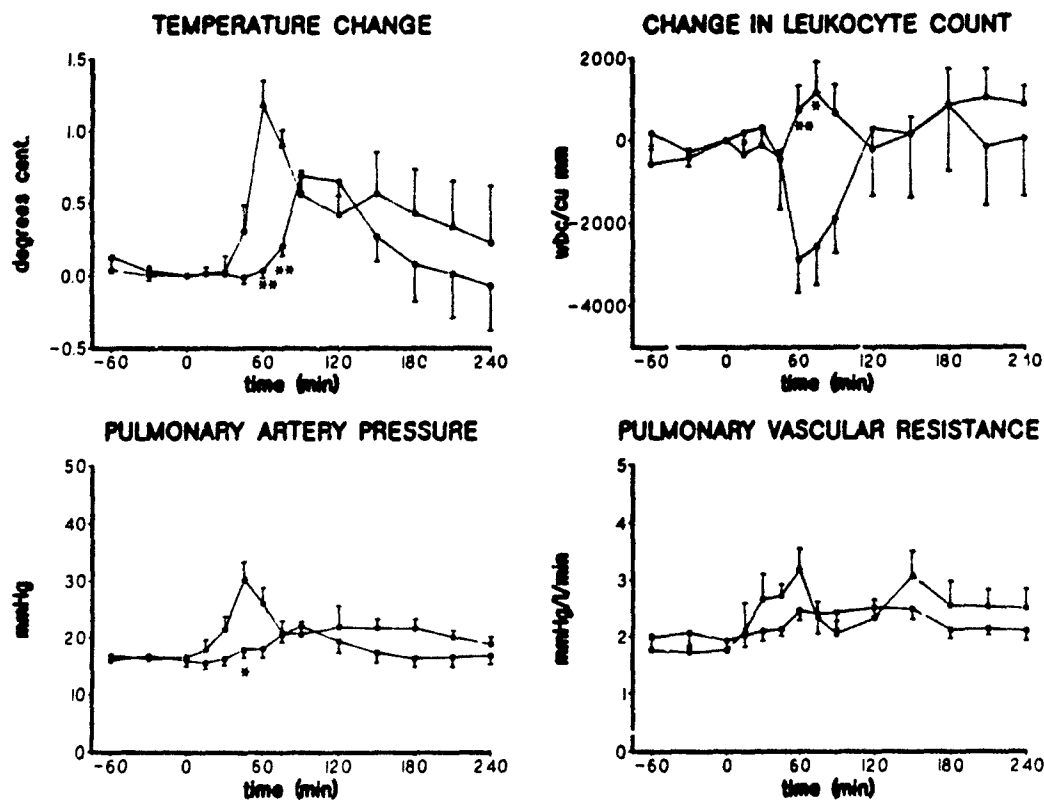


FIG. 6. Pyrogenic response, leukocyte count, pulmonary artery pressure, and pulmonary vascular resistance in awake sheep after intravenous injection of LPS (20 ng/kg in saline) from *S. marcescens* ($n = 5$) (○) or after preincubation with a 200-fold excess of LALF ($n = 4$) (●). Points which significantly differ at $P < 0.05$ and $P < 0.001$ are indicated with a star and double star, respectively.

120:1 ratio of LALF to LPS by weight decreased many toxic effects of LPS in a rabbit model of meningococcal lipooligosaccharide challenge (2). Since the molecular weight of LALF is greater than that of polymyxin B, the potency of LALF is presumably similar to or greater than that of polymyxin B in neutralizing LPS in animal models when estimated on a molar basis.

In our precipitation assays using ^3H -LPS, we found that a concentration of 250 to 700 μg of LALF per ml was needed for 50% binding of LPS in solutions ranging from 2.3 to 8.5 μg of LPS per ml. This corresponded to binding ratios (LALF to LPS) varying from 52:1 to 226:1 for the six LPSs tested. Although these ratios are similar to what we found was necessary for the neutralization of LPS, they are not strictly comparable; the preparations of LPS, the methods of LPS standardization, and experimental conditions that we utilized in the binding assays were not identical to those in the neutralization assays.

Prior studies have reported that LALF is more efficient at inhibiting the bacterial growth of rough gram-negative strains than smooth strains (22), that complete LPS but not purified O polysaccharide chains inhibit the ability of LALF to hemolyze erythrocytes sensitized with LPS (29), and that LALF inhibits endothelial cell stimulation by rough and smooth LPS from several different gram-negative strains (11). These studies suggest that LALF binds to the core glycolipid region of LPS. Our findings that LALF binds LPS from rough mutant *E. coli* J5 in addition to LPS from strains with complete O polysaccharide side chains, and that heterologous LPS competes for the binding of smooth LPS, are in agreement with these results.

The ability of LALF to neutralize LPS in a variety of biological assays raises the possibility that LALF, or an analog, might be therapeutically effective as an antiendotoxin. Because our supply of LALF was limited, all of our in vivo experiments were performed by preincubating LPS with LALF before injection. Further testing, with greater quantities of LALF given to animals before and after LPS challenge, will be required to learn whether LALF is protective in these conditions, and if so, to measure its toxicity and determine a therapeutic ratio. The report by Alpert and colleagues that LALF improves multiple physiological parameters, lowers endotoxin levels, and increases survival in a rabbit model even when it is administered to the rabbits 30 min after meningococcal lipooligosaccharide challenge is particularly encouraging (2). Further studies are also needed to assess whether LALF can bind and neutralize the physicochemical forms of LPS which are released from the bacterial surface into the bloodstream. Since LALF was able to neutralize many different activities of LPS in several animal species, such experiments seem warranted.

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SOUNDING BOARD

ANTI-ENDOTOXIN MONOCLONAL ANTIBODIES

SEVERAL monoclonal antibodies directed against bacterial lipopolysaccharide (endotoxin) are being developed for the adjunctive treatment of gram-negative sepsis. Two of these, E5 (XOMA, Berkeley, Calif.)¹ and HA-1A (Centocor, Malvern, Pa.),² have been studied in clinical trials and evaluated by the Food and Drug Administration for use in the United States. An open advisory-committee meeting was held by the FDA on September 4, 1991, at which new information was presented about the preclinical and clinical studies of these two products.³ This article discusses some of the issues relating to the development and evaluation of these agents in the context of this new information. We focus on HA-1A because the analysis by the FDA of recently submitted data on E5 was not complete at the time of the meeting. On the basis of currently available preclinical and clinical data, we believe that a second placebo-controlled clinical trial of HA-1A is warranted to determine whether it should be widely used.

PRECLINICAL STUDIES

Unfortunately, there are few published data on E5 and HA-1A. The reports leave unanswered many questions concerning the epitopic specificity, binding characteristics, and biologic effects of these antibodies.

E5 is a murine IgM monoclonal antibody, raised in mice immunized with *Escherichia coli* J5, that binds to an epitope on lipid A.¹ It has been reported to bind to heterologous smooth lipopolysaccharide.^{1,4,5} Two reports have addressed the ability of E5 to protect animals against endotoxin or bacterial challenge. In one study, E5 only minimally diminished the physiologic responses to lipopolysaccharide in sheep.⁶ In another study, E5 added only slightly to the protection pro-

vided by antibiotics in mice challenged with gram-negative bacteria.⁷

HA-1A is a human IgM monoclonal antibody that binds to lipid A.^{2,8,9} It was derived from a heterohybridoma created from the spleen cells of a patient who had been vaccinated with *E. coli* J5 before splenectomy.¹⁰ The IgM produced by this hybridoma was initially described as binding specifically to a broad spectrum of smooth lipopolysaccharides and bacteria in an enzyme-linked immunosorbent assay.¹⁰ Subsequent reports have suggested that HA-1A binds poorly to smooth lipopolysaccharide in such assays,^{8,11} but that it binds to some types of smooth lipopolysaccharide in fluid phase, as measured by rate nephelometry.⁹ This technique, however, may detect low-affinity as well as high-affinity interactions between antibody and antigen. A recent report⁸ and information presented at the FDA meeting⁹ suggest that HA-1A binds only slightly to smooth bacteria that have not been exposed to antibiotics. A different group found that IgM purified from the same hybridoma did bind to gram-negative bacteria, but that it also bound to gram-positive bacteria, fungi, cardiolipin, and lipoproteins, raising doubt about its specificity.¹¹ There is very little information on the ability of HA-1A to neutralize lipopolysaccharide in functional in vitro assays. Discussion at the FDA meeting suggested that it may decrease the lipopolysaccharide-induced production of tumor necrosis factor in cells obtained from leukapheresis packs, but not in whole blood,¹² a more physiologic assay.

Five studies have been published on the ability of the IgM produced by this hybridoma to protect against lipopolysaccharide challenge in animal models. The initial study reported that the hybridoma culture medium protected mice from lethal bacteremia and rabbits from the dermal Schwartzman reaction.¹⁰ A subsequent abstract indicated that the IgM decreased mortality among neutropenic rabbits with pseudomonas bacteremia.¹³ However, the same IgM provided only moderate protection from lipopolysaccharide-induced lung injury in rats¹⁴ and did not prevent lipopolysaccharide-induced hypotension in rabbits.¹⁵ Other investigators were unable to reproduce the protective effect against the dermal Schwartzman reaction or to detect protection in sensitized mice treated with an IgM prepared from the same hybridoma.¹⁶ The ability of HA-1A to protect animals from endotoxin challenge was also discussed in the FDA meeting. It was noted by a company representative¹⁷ that

the results are not consistently reproducible over time and from laboratory to laboratory. This lack of reproducibility has troubled workers in the field of anti-endotoxin antibodies for many years and it leads us to the conclusion that these models would not be reliable as routine potency and release assays.

Thus, two problems involving the preclinical studies of HA-1A have become apparent. First, the data on binding that were presented in the initial description of the antibody¹⁰ and endorsed in the published

study² are substantially different from those more recently described by the company,⁸ by participants at the FDA open meeting,⁹ and by other investigators.¹¹ Second, there is no experimental model in which HA-1A has consistently protected animals from endotoxic challenge.¹⁷ These difficulties seriously erode the stated rationale for the clinical study² and underscore the fact that the premise on which this approach is based remains unproved and unclear.^{11,18} They also bring out the practical problem that there is no established method to ensure quality control of the antibody, since the characteristics of HA-1A that are related to protection are unknown.

CLINICAL STUDIES

E5 has been tested in two placebo-controlled clinical trials. In the first trial,¹ 486 patients with signs of gram-negative infection and a systemic septic response were enrolled. The administration of E5 was associated with increased survival only in the relatively small number of patients with gram-negative sepsis who were not in refractory shock (137 patients). In this subgroup of patients, 43 percent of those given placebo and 30 percent of those given E5 died within 30 days after treatment ($P = 0.01$). A second large trial (847 patients) was then conducted to test the hypothesis that E5 benefits patients with gram-negative sepsis who are not in refractory shock.¹⁹ E5 did not improve survival in the 530 patients with documented gram-negative sepsis. A trend toward improved survival was observed in a subgroup of patients with major-organ failure without refractory shock (139 patients), but a detailed independent analysis of these data has not been presented.

HA-1A has been studied in a single, randomized, placebo-controlled clinical trial of patients with presumed gram-negative sepsis and was reported to prevent mortality in a subgroup of patients who had gram-negative bacteremia, whether or not they were in shock.² Although this investigation was carefully designed, questions have arisen concerning the demonstration of therapeutic efficacy, and the data analysis presented at the FDA meeting²⁰ differed from that reported by Ziegler et al.²

Our concern, which we discuss here, is that a significant result was found in only one of many overlapping subgroups; that the statistical result was marginal; that a protective effect was seen only at clinical centers with high mortality and only in patients with shock; that the APACHE II system used to stratify patients may have been inappropriately applied; that patients who received inadequate or unknown antibiotic treatment were included in the analysis; and that the data were not stratified according to the time elapsed before the antibody or placebo was infused.

Patient subgroups in the HA-1A trial were clearly defined in advance of analysis.²⁰ Anticipated covariates were specified and provisions were described for making certain key judgments (such as whether or not a patient had received adequate antimicrobial therapy,

or whether a death was unrelated to sepsis) before the study code was broken. The three primary subgroups used in the analysis of efficacy were (in order of importance) patients with gram-negative sepsis, who had documented infection with gram-negative organisms (with or without bacteremia) but not infection with other microbes; patients with gram-negative bacteremia, who had positive blood cultures for gram-negative bacteria, whether or not they had positive cultures for other microbes; and patients with gram-negative infection, who consisted of all patients with gram-negative disease, regardless of other kinds of ongoing infection. In addition, two categories of mortality (mortality due to sepsis and mortality due to all causes) were analyzed for two times after infusion (at 14 days and over a 28-day period). Mortality due to sepsis was identified as the more important indicator of efficacy.²⁰ The FDA analysts suggested that because multiple comparisons were made in the analysis (three subgroups, two categories of mortality, and two observation periods), the level of statistical significance should be adjusted: for a statistically significant difference, it was recommended that the necessary P value should be below a level that was somewhere between 0.01 and 0.03.²¹

According to these guidelines, HA-1A was found to be associated with a beneficial outcome in only one of the three subgroups in the efficacy analysis (patients who had gram-negative bacteremia) and in relation to only one of the end points (mortality from all causes over the 28-day period)²⁰ (Table 1). During the last two weeks of the trial, four deaths that were not due to sepsis occurred in the placebo group; excluding these deaths from the analysis raised the P value from 0.014 (the P value for mortality due to all causes) to 0.039 (the P value for mortality due to sepsis), which was not within the estimated range of values that would show a statistically significant result.²¹ The drug was not effective in the subgroup with gram-negative sepsis. Furthermore, among the 201 patients with non-bacteremic gram-negative infections, mortality was somewhat higher in the HA-1A group than in the placebo group at both 14 and 28 days.²²

Data were also presented at the FDA meeting regarding the effect of HA-1A in subgroups of patients with gram-negative bacteremia, according to the presence or absence of shock²³ (Table 2). The published report, in which analysis was based on a Cox proportional-hazards model, stated that "HA-1A reduced mortality in both patients with shock and patients without shock."² In contrast, the FDA, analyzing the same data according to different statistical methods, came to the following conclusion: "in the no-shock group there was not a significant difference in mortality. . . . If you look over time, those curves cross. At some time periods it is higher in the treatment group and, in others, in the placebo. There does not appear to be a significant difference."²³ Although the study was not primarily designed to examine this issue, these data suggest that the benefit of HA-1A among patients with gram-negative bacteremia may be limited

to patients with gram-negative bacteremia who are in shock.

Patients who receive inadequate antimicrobial chemotherapy present a special problem in trials of anti-endotoxin antibodies. Anti-endotoxin monoclonal antibodies are usually thought to be adjunctive therapy; they should probably not be expected to benefit patients who receive inadequate antimicrobial chemotherapy. This point would seem to apply particularly to HA-1A, since the available data suggest that HA-1A binds only slightly to bacteria that have smooth lipopolysaccharide (i.e., most blood isolates), unless the bacteria have been treated with antibiotics.^{8,9} In the placebo group of the HA-1A trial, inappropriate antimicrobial therapy was strongly associated with death in patients with gram-negative bacteremia (mortality of 69 percent with inappropriate therapy and 27 percent with appropriate therapy).²⁴ Patients who received inadequate antimicrobial chemotherapy were not excluded from the analysis of the HA-1A trial; instead, patients with inadequate or unknown antimicrobial therapy were included in the multivariate analysis (16 patients in the placebo group [17 percent] and 10 in the HA-1A group [10 percent]).²⁰ When only patients who received adequate antimicrobial therapy were considered in the analysis of mortality after 14 days, there was no significant difference between the two groups of patients with gram-negative bacteremia (21 deaths among 79 patients in the placebo group and 20 deaths among 95 patients in the HA-1A group [data from slides presented at the FDA meeting (Siegel JP)]²⁰). Data were not presented at the FDA meeting concerning deaths at the 28-day end point.

The HA-1A clinical trial took place at 22 study sites. Analysis of the consistency of the drug effect at the sites where at least one patient with gram-negative bacteremia was included in each study group and at least one patient died indicated that many more study sites found lower mortality in the HA-1A group than found it in the placebo group (11 sites vs. 1 site).²⁵ This apparent consistency among study sites may be important evidence that the effect of HA-1A is genuine. However, concern was raised at the FDA meeting about another feature of the distribution of patients among the study sites. As a panelist²⁶ pointed out,

Six of the 22 sites had more than 50-percent mortality in the placebo group and 16 of them had less than that. . . . All of the effect is in the high-mortality sites within the gram-negative bacteremia group, so that there is a 64-percent mortality in the placebo group and 22 percent in the HA-1A, whereas, actually, there is a slight advantage to placebo in the low-mortality sites.

The ensuing discussion did not explain why HA-1A might have its effect principally in patients at centers that had high case fatality rates for gram-negative bacteremia.

Table 1. Mortality Due to Sepsis and All Causes in the HA-1A Trial, According to Patient Subgroup.*

SUBGROUP	MORTALITY AT 14 DAYS DUE TO SEPSIS AND ALL CAUSES†		P VALUE	
	PLACEBO	HA-1A	SEPSIS	ALL CAUSES
	patients dead/all patients (%)		day 14†	over 28 days
Gram-negative sepsis	47/145 (32)	40/137 (29)	0.56	0.29
Gram-negative bacteremia	32/95 (34)	25/105 (24)	0.12	0.039
Gram-negative infection	61/207 (29)	56/194 (29)	0.89	0.47

*Adapted from slides presented at the FDA meeting (Siegel JP).²⁰

†Mortality due to sepsis equaled mortality due to all causes at 14 days.

The published clinical studies on E5 and HA-1A^{1,2} both used the APACHE II scoring system²⁷ to stratify patients according to physiologic status and underlying disease severity at the time of entry. Neither investigation fully considered the nonlinear relation between APACHE II score and the risk of hospital death.^{27,28} Instead of using the raw APACHE II score in multivariate analysis, it would have been more informative to calculate and enter the risk of death during hospitalization in each patient. For example, the APACHE II equation that relates a raw score to the risk of in-hospital death gives great weight to emergency surgery as an independent risk factor; depending on the raw APACHE II score, emergency surgery can add as much as 14 percent to the risk of in-hospital death. Using individual risks rather than the raw APACHE II scores in the multivariate analysis might have controlled more accurately for an imbalance in the distribution of major underlying disorders in the HA-1A trial — which, in this instance, favored the HA-1A group.¹⁸ The placebo group contained more patients with disseminated intravascular coagulation (21 percent vs. 18 percent), adult respiratory distress syndrome (13 percent vs. 9 percent), acute hepatic failure (26 percent vs. 19 percent), acute renal failure (46 percent vs. 35 percent), and recent surgery (34 percent vs. 29 percent).²

The published report describing the HA-1A trial indicated that "The median [emphasis added] intervals between the diagnosis of sepsis and infusion of the study drug were 9.3 hours in the placebo group and 14.3 hours in the HA-1A group."² It was stated in the FDA meeting that the mean interval before infusion in

Table 2. Mortality due to Sepsis in Patients with Gram-Negative Bacteremia, According to the Presence or Absence of Shock.*

	MORTALITY AT DAY 14		MORTALITY OVER 28 DAYS	
	PLACEBO	HA-1A	P VALUE	P VALUE
	patients dead/all patients (%)			
All patients	32/95 (34)	25/105 (24)	0.12	0.039
Patients with shock	23/48 (48)	13/54 (24)	0.012	0.023
Patients without shock	9/47 (19)	12/51 (24)	0.60	—

*Adapted from slides presented at the FDA meeting (Siegel JP).²⁰

both groups was approximately 20 hours,²⁹ indicating that many of the patients received HA-1A or placebo a very long time after the onset of sepsis. Unfortunately, there are no available data that reveal the efficacy of the drug according to the interval between the onset of sepsis or shock and the infusion. This information is of obvious importance to clinicians. The possible role of differences in the time of administration of HA-1A or placebo in determining the outcome of the trial is also impossible to evaluate without further data. Nevertheless, the time of drug administration may be a very important determinant of clinical outcome. According to the FDA analyst, "on the treatment day and the day immediately thereafter, is where the largest evidence of effect and most of the difference between the treatment groups occurred."³⁰ During this period, 13 percent of the patients given placebo died, as compared with 5 percent of those given HA-1A.³⁰ Much more information is needed about this aspect of the HA-1A trial.

CONCLUSION

Although there are few data available on preclinical studies of E5, the available data from the two clinical trials suggest that E5 does not reproducibly prevent mortality in patients with gram-negative sepsis, even in those who are not in refractory shock. A more complete picture may emerge when the results of the second E5 study are fully analyzed.

In our opinion, the evidence that HA-1A reduces mortality among patients with gram-negative bacteremia is suggestive but not conclusive. A significant result was found in only one of many possible subgroup-end-point categories, and HA-1A was not shown to prevent death due to the condition that it was intended to treat — gram-negative sepsis. Post hoc analysis of prospective studies should always be interpreted cautiously. Nevertheless, the flaws in the scientific foundation used to justify a clinical trial of HA-1A, the uncertainties about the analysis of that trial, and the marginal statistical significance of the result ("borderline," according to a statistical consultant to the FDA³¹) persuade us that the null hypothesis has not been convincingly rejected in the HA-1A study. An accurate determination of efficacy seems especially important for HA-1A: even if the drug is used according to the criteria for study entry, it will potentially benefit a very small fraction of the patients who receive it, and its cost is substantial (currently over \$3,500 per dose in Europe). The use of such an expensive therapy in an appropriate and cost-effective manner requires conclusive knowledge of its efficacy.

The inconsistent outcomes of the two E5 trials,^{1,19} as well as previous experience in the evaluation of corticosteroid therapy for sepsis,³²⁻³⁵ support the view that it is difficult to test a therapeutic agent in this complex population and obtain the same result twice. It should be noted that the HA-1A trial is not a confir-

mation of the earlier trial of polyclonal antiserum to *E. coli* J5³⁶; HA-1A is a new agent, and the protective factor in the polyclonal antiserum remains unknown and controversial. Accordingly, we believe that the use of HA-1A should remain experimental until a second randomized, placebo-controlled trial has confirmed its efficacy. This trial would test the treatment hypothesis generated by the first trial — that HA-1A increases survival when given early to patients with presumed gram-negative bacteremia who are in septic shock.

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