

Assessment of Ability of Murine and Human Anti-lipid A Monoclonal Antibodies to Bind and Neutralize Lipopolysaccharide

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Summary

The use of monoclonal antibodies (mAbs) directed to lipid A for the therapy of gram-negative sepsis is controversial. In an attempt to understand their biologic basis of action, we used a fluid-phase radioimmunoassay to measure binding between bacterial lipopolysaccharide (LPS) and two IgM mAbs 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 used, 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 LPS in vitro. Despite large molar excesses, neither antibody neutralized LPS as assessed by the limulus lysate test, by a mitogenic assay for murine splenocytes, or by the production of cytokines interleukin (IL)-1, IL-6, or tumor necrosis factor from human monocytes in culture medium or in whole blood. Our experiments do not support the hypothesis that either of these anti-lipid A mAbs function by neutralizing the toxic effects of LPS.

In 1968, Chedid et al. (1) reported that the passive infusion of antiserum raised to killed rough mutant bacteria protected mice against challenge with heterologous gram-negative bacteria. 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, *Escherichia coli* J5 and *Salmonella 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 Ig directed against common epitope(s). First, purified Ig fractions prepared from the polyclonal antisera protected in animal models (4, 11, 12). Second, the protective activity in

the antisera could be adsorbed 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 Ig in these antisera cross-reacted with LPS from multiple gram-negative strains, and therefore was able to "cross-protect" against heterologous strains. Cross-reactive Ig was hypothesized to protect by neutralizing the toxic effects of endotoxin (10).

Several investigators have generated mAbs 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 mAb that binds to 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-d 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 mAb 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 LPS, 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 nonspecific interactions and the amphipathic nature of LPS. Accordingly, we used a fluid-phase RIA 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 used. 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 mAbs function by binding and neutralizing the toxic effects of LPS.

Materials and Methods

LPS

Unlabeled LPS from *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Serratia marcescens*, *E. coli* J5, and *E. coli* K12,D31m4 (Re) were purchased from List Biol. Laboratories, Inc. (Campbell, CA). Unlabeled LPS from *E. coli* O113 was prepared by the hot phenol method as described by Rudbach et al. (28). Unlabeled LPS from *Neisseria meningitidis* was the kind gift of Martine Caroff (Unité Associée Centre National de la Recherche Scientifique 1116, Orsay, France).

Cultures of *E. coli* O111:B4, *E. coli* O18, and *E. coli* J5 were the kind gifts of Dr. David Morrison (University of Kansas Medical Center, Kansas City, KS), 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).

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 OD 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 et al. (31). The concentrations of LPS were estimated by a spectrophotometric limulus amoebocyte lysate (LAL)¹ gelation assay using an *E. coli* O113 LPS standard containing 10 endotoxin U/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 µg/ml and cpm/µg were calculated by counting a 0.4-ml volume combined with 4.5 ml of optiflor scintillation fluid (Packard Instrument Co., 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; *E. coli* O6, 7,200; *E. coli* O7, 12,150; *E. coli* O8, 10,990; *E. coli* O16, 17,100; *E. coli* O18, 6,150; *E. coli* O25, 11,040; *E. coli* O75, 4,700; and *E. coli* O111:B4, 4,040. Greater than 99% of each radiolabeled LPS was demonstrated to remain in the water phase after 1:1 ether/water extraction at pH 5. SDS-PAGE 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 et al. (33). This LPS contained 22,000 cpm/µg.

Antibodies and Controls

The human anti-lipid A IgM mAb (HA-1A, Centoxin) used in the study was from Centocor BV. (Leiden, The Netherlands). It was supplied as a 5 mg/ml solution containing 5% human serum albumin. Murine anti-lipid A IgM mAb (E5) was the kind gift of Dr. Robert Rubin (Infectious Disease Unit, Massachusetts General Hospital). It was supplied as a 2 mg/ml solution in PBS, pH 7.3. Murine IgG2a mAb 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 RIA

Radiolabeled LPS was incubated in dilutions of each mAb 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 µg/ml of each smooth LPS tested was incubated in 150 µl of dilutions of the mAb to be tested in .01 M PBS, pH 7.3 for 120 min at 37°C in a 1.5-ml 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. After incubation, the solution was cooled on ice for 15 min. An equal volume of iced saturated ammonium sulfate was then added dropwise and the solution was allowed to sit at 4°C for another 15 min. The tubes were next centrifuged at 12,000 g for 15 min. Supernatants were carefully aspirated, and pellets were washed twice with 50% ammonium sulfate and resuspended in 300 µl of PBS.

¹ Abbreviations used in this paper: HSA, human serum albumin; LAL, limulus amoebocyte lysate.

The quantities 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 Instrument Co.). 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 after precipitation with 50% ammonium sulfate as assessed by a capture IgM ELISA technique. Greater than 80% of E5 was found in the pellet after precipitation as assessed by a protein assay using bicinchoninic acid (Pierce Chemical Co., Rockford, IL). Less than 5% of each smooth LPS tested precipitated in PBS alone. However, >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 >85% using this assay. At low protein concentrations (<50 µg/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 percent recovered cpm as determined by the formula: 100 × (cpm in the pellet/cpm recovered), expressed to the nearest whole percentage point. Results are given as the mean of at least two experiments.

LAL Assay

A spectrophotometric LAL assay was used as previously described (35). Briefly, 50 µl of a solution of 20 µg/ml of each mAb in pyrogen-free saline were incubated with 50 µl of dilutions of LPS for 30 min at 37°C in a 96-well microtiter plate. 100 µl of LAL were then added, and the plate was incubated at 37°C for an additional 60 min. 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 with coagulation of the LAL in saline alone.

Mitogenic Assay

The mitogenic assay was performed essentially as described by Jacobs et al. (36). Briefly, dilutions of LPS were preincubated in the presence of 625 µg/ml mAb or control, or 50 µg/ml polymyxin B for 2 h at 37°C. This solution was then diluted in RPMI media supplemented with antibiotics containing 0.01 M HEPES and 10% FCS and incubated in a volume of 200 µl for 48 h with 5 × 10⁶ spleen cells from BALB/c mice. The final antibody or HSA control concentrations in culture were 25 µg/well and the final polymyxin B concentration in culture was 2 µg/well. 1 µCi of [³H]thymidine was next added to each well, and cells were incubated for an additional 16 h. Incorporated radioactivity was measured using a cell harvester (Cambridge Technology, Inc., 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. PBMC were obtained by centrifugation on Ficoll (MSL; Eurobio, Les Ulis, 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 antilymphocyte antibodies, OKT11 (Ortho Diagnostics, Inc., Raritan, NJ) and IOBI (Immunotech, Marseille, France). Human mononuclear adherent cells (5 × 10⁵ nonspecific esterase-positive cells per well), cultured in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 100 IU of penicillin per ml and 100 µg of streptomycin per ml, were incubated for 24 h in the presence of free LPS, or in the presence of LPS that had been incubated previously with dilutions of anti-lipid A antibodies or 2 µg/ml polymyxin B for 1 h at 37°C in culture medium containing 3.3% decomplexed 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 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 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 in whole blood was measured as described (39). Briefly, 500 µl 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. Preincubations (LPS alone or with antibodies or polymyxin B) were performed in the presence of 3.3% decomplexed normal human serum as described above. Final reaction mixtures contained the indicated amounts of antibody and LPS. Cytokines were assayed after 24 h of culture.

TNF Assay. An RIA specific for TNF-α was performed according to Munoz et al. (37) with minor modifications. Briefly, on day 1, 100 µl 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 µl of standards or samples. To determine the nonspecific binding, 100 µl of BSA buffer was added to a tube instead of sample. Then 300 µl of BSA buffer was added to each tube. After vortexing, the tubes were incubated for 24 h at room temperature. Standards of TNF-α (Rhone Poulenc, Vitry-sur-Seine, France) containing 0, 40, 80, 150, 300, 600, 1,250, 2,500, 5,000, and 10,000 pg/ml were employed. On day 2, 100 µl of a solution of ¹²⁵I-TNF-α (30 µCi/µg, New England Nuclear, Boston, MA) containing ≈10,000 cpm was added to each tube. The tubes were vortexed and incubated at room temperature for 24 h. On day 3, 500 µl 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 percent 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.

IL-1 Assay. A similar RIA protocol as that used for TNF-α measurement was employed to determine concentrations of IL-1β. Standard IL-1β was obtained from Rhone Poulenc. Rabbit anti-IL-1β antiserum (1:150) was purchased from Endogen, Inc. (Boston, MA), and ¹²⁵I-IL-1β (126–253 µCi/µg) was obtained from New England Nuclear. 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.

The IL-1 α concentrations were determined by ELISA using two anti-IL-1 α mAbs as described (37). Briefly, on day 1, ELISA microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with 100 μ l of monoclonal mouse IgG1 anti-rhIL-1 α (10 μ g/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 μ l 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 μ l of peroxidase-conjugated anti-mouse IgG2b (1:5,000) (Southern Biotechnology 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 dihydrochloride (Sigma Chemical Co.) and hydrogen peroxide (0.06%). The reaction was stopped with 50 μ l of 3N HCl, and the absorbance was read at 492 nm on 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 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, Belgium). 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-ME (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. 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% 1.0 N HCl, 2.5% of

80% acetic acid, pH 7.4). After overnight incubation at 37°C, OD 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 LPS 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 mAbs to compare binding with the O-specific IgG antibody and to see if higher concentrations of anti-lipid A mAbs would be more efficient at binding. These results are shown in Fig. 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, whereas HA-1A bound somewhat less. As expected, the O-specific IgG mAb bound homologous LPS efficiently at low concentrations (half-maximal binding at 5–10 μ g/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 Fig. 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 (data 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 mAb was observed. Each ex-

Table 1. Percentage of [3 H]LPS Bound by Different Ig Preparations at 200 μ g/ml*

[3 H]LPS (5 μ /ml)	Anti- <i>E. coli</i>		Polyclonal	Human IgM	HA-1A/HSA	Polyclonal	
	PBS/HSA	O111:B4/HSA	Human IgM/HSA	myeloma/HSA		Mouse IgM/HSA	E5/HSA
<i>E. coli</i> O1	3.8 \pm 3.3	1.5 \pm 0.7	9.7 \pm 2.1	7.5 \pm 3.5	26.2 \pm 10.4	23.0 \pm 0.0	11.0 \pm 1.4
<i>E. coli</i> O2	3.5 \pm 3.5	1.0 \pm 0.0	42.0 \pm 18.3	3.5 \pm 2.1	60.7 \pm 22.4	59.5 \pm 3.5	8.0 \pm 1.4
<i>E. coli</i> O4	2.5 \pm 1.2	2.0 \pm 1.4	5.0 \pm 0.0	4.0 \pm 1.4	15.1 \pm 8.7	7.5 \pm 3.5	5.0 \pm 0.0
<i>E. coli</i> O6	1.2 \pm 0.4	1.5 \pm 0.7	2.0 \pm 0.0	2.0 \pm 0.0	7.5 \pm 3	6.5 \pm 6.4	4.0 \pm 1.4
<i>E. coli</i> O7	1.3 \pm 0.7	1.0 \pm 0.0	1.5 \pm 0.7	1.5 \pm 0.7	10.5 \pm 7.4		
<i>E. coli</i> O8	1.8 \pm 1.1	1.0 \pm 0.0	2.0 \pm 1.4	3.0 \pm 0.0	13.0 \pm 3.6		
<i>E. coli</i> O18	1.4 \pm 0.7	1.0 \pm 0.0	2.0 \pm 0.0	1.5 \pm 0.7	9.8 \pm 6.7		
<i>E. coli</i> O25	2.0 \pm 1.2	1.0 \pm 0.0	7.0 \pm 6.2	2.5 \pm 0.7	16.1 \pm 9.2	3.0 \pm 0.0	1.6 \pm 0.5
<i>E. coli</i> O75	2.1 \pm 1.7	1.5 \pm 0.7	6.0 \pm 4.3	1.5 \pm 0.7	11.2 \pm 5.5	5.5 \pm 2.1	3.6 \pm 2.1
<i>E. coli</i> O111:B4	6.4 \pm 5.8	65.0 \pm 15.0	4.0 \pm 1.4	5.0 \pm 1.4	23.2 \pm 8.5	3.0 \pm 0.0	6.5 \pm 7.8

* Each value represents mean \pm SD.

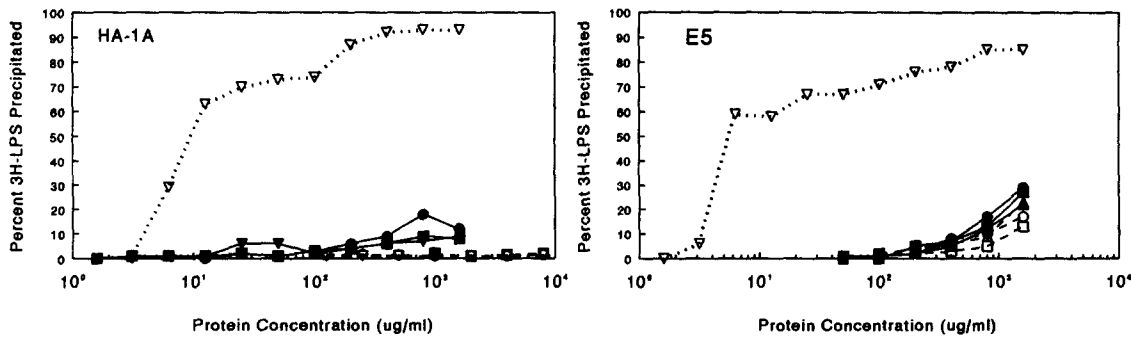


Figure 1. Binding of HA-1A (left) and E5 (right) to 5 µg/ml LPS from several smooth gram-negative strains as a function of concentration. Background was subtracted from each point on the curves, and each curve is the mean of two experiments. (Solid symbols, solid lines) HA-1A and E5. (Open symbols, dashed lines) HSA control (left) and murine IgM control (right). (Triangles) *E. coli* O4; (circles) *E. coli* O25; (squares) *E. coli* O75; and (inverted triangles) *E. coli* O111:B4. A single experiment showing binding of an IgG mAb 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.

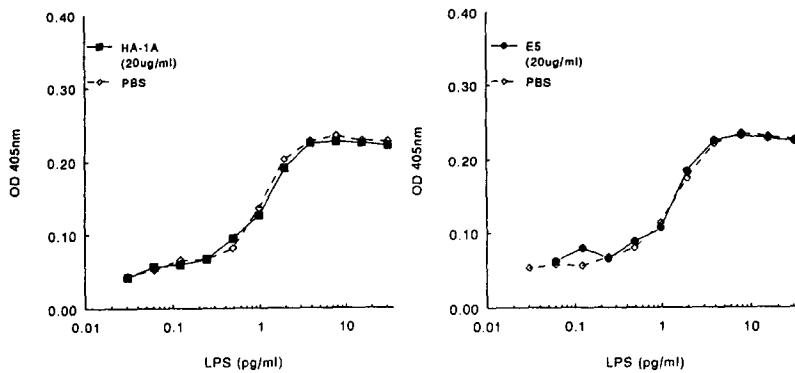


Figure 2. Effect of HA-1A (left) and E5 (right) on LPS-induced activation of LAL. Each mAb was preincubated with the indicated concentrations of LPS from *E. coli* J5 before incubation with limulus lysate. OD of reaction was measured after 1 h.

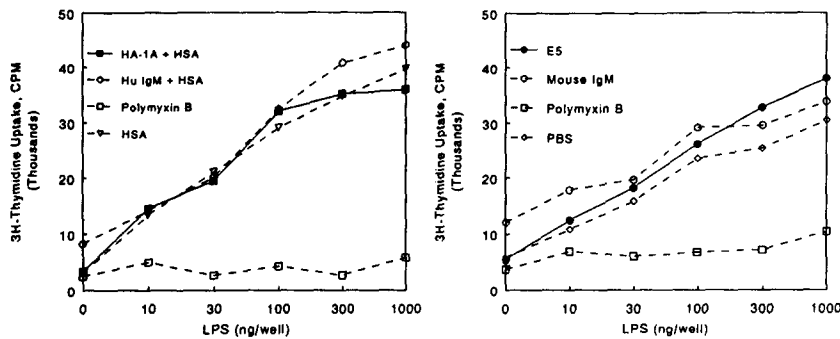


Figure 3. Effect of HA-1A (left) and E5 (right) on LPS-induced mitogenic activity on splenocytes. Wells contained indicated amounts of LPS from *E. coli* O18 and 25 µg antibody or 25 µg polymyxin B or PBS.

periment 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 nor 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 Fig. 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 was then assessed as described in Materials and 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 Figs. 4 and 5, respectively. Representative experiments for E5 ($n = 2$, adherent cells; $n = 4$, whole blood) are shown in Fig. 6.

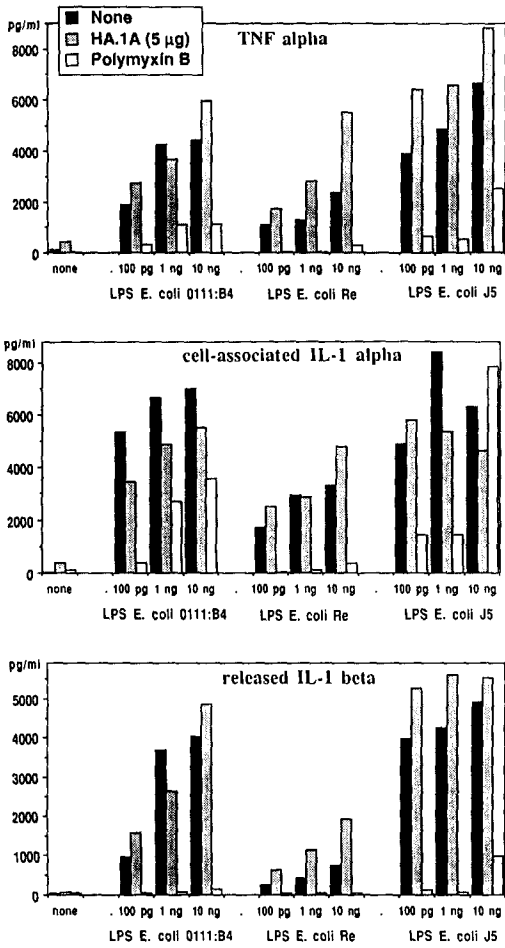


Figure 4. Effect of HA-1A on LPS-induced stimulation of TNF- α , cell-associated IL-1 α , and IL-1 β from adherent human monocytes. HA-1A had no effect on LPS-induced stimulation of IL-6 in this or other experiments. Wells contained indicated amounts of LPS and antibody or 1 μ g polymyxin B.

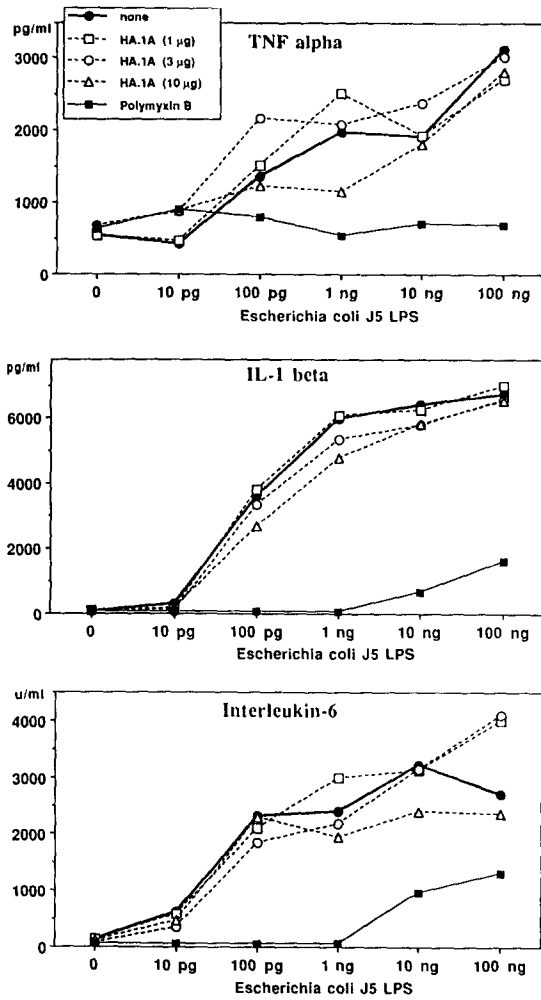


Figure 5. Effect of HA-1A on LPS-induced stimulation of TNF- α , IL-1 β , and IL-6 in whole blood assay. Wells contained indicated amounts of LPS and antibody or 1 μ g polymyxin B.

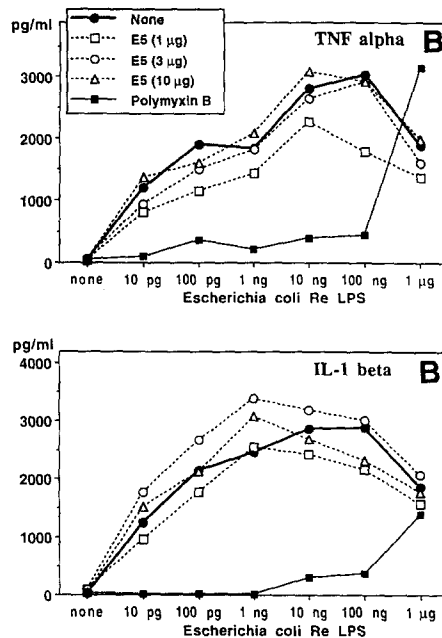
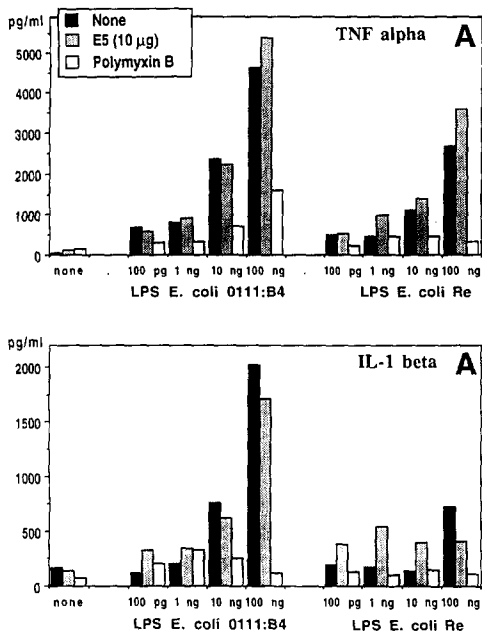


Figure 6. Effect of E5 on LPS-induced stimulation of TNF- α and IL-1 β from adherent human monocytes (A), and in whole blood assay (B). Wells contained indicated amounts of LPS and antibody or 1 μ g polymyxin B.

Discussion

These studies demonstrate that anti-lipid A mAbs 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 mAbs 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 nonspecific binding to the solid-phase matrix, we used a fluid-phase RIA 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 use 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 μ g/ml of an IgG mAb specific for the O-polysaccharide of this LPS. The RIA that we used 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 >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 nonspecific. It has been suggested that anti-core glycolipid antibodies may interact with LPS in a nonspecific 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 nor E5 was able to neutralize the effects of LPS in any of the assays we used, 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 mAbs 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 \sim 90-fold less than this based on 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. Because there are 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 used 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.

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References

- Chedid, L., M. Parant, and F. Boyer. 1968. A proposed mechanism for natural immunity to enterobacterial pathogens. *J. Immunol.* 100:292.
- Braude, A.I., and H. Douglas. 1972. Passive immunization against the local swartzman reaction. *J. Immunol.* 108:505.
- Braude, A.I., H. Douglas, and C.E. Davis. 1973. Treatment and prevention of intravascular coagulation with antiserum to endotoxin. *J. Infect. Dis.* 128:S157.
- Davis, C.E., E.J. Ziegler, and K.F. Arnold. 1978. Neutralization of meningococcal endotoxin by antibody to core glycolipid. *J. Exp. Med.* 147:1007.
- McCabe, W.R. 1972. Immunization with R mutants of *S. minnesota*. I. Protection against challenge with heterologous gram-negative bacilli. *J. Immunol.* 108:601.
- Johns, M.A., A. Skehill, and W.R. McCabe. 1983. Immunization with rough mutants of *Salmonella minnesota*. IV. Protection by antisera to O and rough antigens against endotoxin. *J. Infect. Dis.* 147:57.
- Young, L.S., P. Stevens, and J. Ingram. 1975. Functional role of antibody against "core" glycolipid of *Enterobacteriaceae*. *J. Clin. Invest.* 56:850.
- Greisman, S.E., J.R. DuBuy, and C.L. Woodward. 1978. Experimental gram-negative bacterial sepsis: reevaluation of the ability of rough mutant antisera to protect mice. *Proc. Soc. Exp. Biol. Med.* 158:482.
- Greisman, S.E., and C.A. Johnston. 1988. Failure of antisera to J5 and R595 rough mutants to reduce endotoxemic lethality. *J. Infect. Dis.* 157:54.
- Ziegler, E.J., J.A. McCutchan, J. Fierer, M.P. Glauser, J.C. Sadoff, H. Douglas, and A.I. Braude. 1982. Treatment of gram-negative bacteremia and shock with human antiserum to a mutant *Escherichia coli*. *N. Engl. J. Med.* 307:1225.
- Ziegler, E.J., J.A. McCutchan, H. Douglas, and A.I. Braude. 1975. Prevention of lethal *Pseudomonas* bacteremia with epimerase-deficient *E. coli* antiserum. *Trans. Assoc. Am. Physicians.* 88:101.
- McCabe, W.R., A. DeMaria, H. Berberich, and M.A. Johns. 1988. Immunization with rough mutants of *Salmonella minnesota*: protective activity of IgM and IgG antibody to the R595 (Re chemotype) mutant. *J. Infect. Dis.* 158:291.
- Marks, M.I., E.J. Ziegler, H. Douglas, L.B. Corbeil, and A.I. Braude. 1982. Induction of immunity against lethal *Haemophilus influenzae* type b infection by *Escherichia coli* core lipopolysaccharide. *J. Clin. Invest.* 69:742.
- Sakulramrung, R., and G.J. Domingue. 1985. Cross-reactive immunoprotective antibodies to *Escherichia coli* O111 rough mutant J5. *J. Infect. Dis.* 151:995.
- Teng, N.N.H., H.S. Kaplan, J.M. Hebert, C. Moore, H. Douglas, A. Wunderlich, and A.I. Braude. 1985. Protection against gram-negative bacteremia and endotoxemia with human monoclonal IgM antibodies. *Proc. Natl. Acad. Sci. USA.* 82:1790.
- Greenman, R.L., R.M.H. Schein, M.A. Martin, R.P. Wenzel, N.R. MacIntyre, G. Emmanuel, H. Chmel, R.B. Kohler, M. McCarthy, J. Plouffe, et al. 1991. A controlled clinical trial of E5 murine monoclonal IgM antibody to endotoxin in the treatment of gram-negative sepsis. *J. Am. Med. Assoc.* 266:1097.
- Nelles, M.J., and C.A. Niswander. 1984. Mouse monoclonal antibodies reactive with J5 lipopolysaccharide exhibit extensive serological cross-reactivity with a variety of gram-negative bacteria. *Infect. Immun.* 46:677.
- Mutharia, L.M., G. Crockford, W.C. Bogard, Jr., and R.E. Hancock. 1984. Monoclonal antibodies specific for *Escherichia coli* J5 lipopolysaccharide: cross-reaction with other gram-negative bacterial species. *Infect. Immun.* 45:631.
- Gigliotti, F., and J.L. Shenep. 1985. Failure of monoclonal antibodies to core glycolipid to bind intact smooth strains of *Escherichia coli*. *J. Infect. Dis.* 151:1005.
- Miner, K.M., C.L. Manyak, E. Williams, J. Jackson, M. Jewell, M.T. Gammon, C. Ehrenfreund, E. Hayes, L.T. Callahan, H. Zweerink, et al. 1986. Characterization of murine monoclonal antibodies to *Escherichia coli* J5. *Infect. Immun.* 52:56.
- Ziegler, E.J., C.J. Fisher, Jr., C.L. Sprung, R.C. Straube, J.C. Sadoff, G.E. Foulke, C.H. Wortel, M.P. Fink, R.P. Dellinger, N.N.H. Teng et al. 1991. Treatment of gram-negative bacteremia and septic shock with HA-1A human monoclonal antibody against endotoxin: a randomized, double-blind, placebo-controlled trial. *N. Engl. J. Med.* 324:429.
- Wenzel, R., R. Bone, A. Fein, R. Quenzer, J. Schentag, K.J. Gorelick, N.I. Wedel, and T. Perl. 1991. Results of a second double-blind, randomized, controlled trial of antiendotoxin antibody E5 in gram-negative sepsis. Program and Abstracts of the 31st Intersci. Conf. Antimicrob. Agents Chemother. pg. 294.
- Ziegler, E.J., N.N.H. Teng, H. Douglas, A. Wunderlich, H.J. Berger, and S. Bolmer. 1987. Treatment of pseudomonas bacteremia in neutropenic rabbits with human monoclonal IgM antibody against *E. coli* lipid A. *Clin. Res.* 35:619A.
- Feeley, T.W., B.D. Minty, C.M. Scudder, J.G. Jones, D. Royston, and N.N.H. Teng. 1987. The effect of human antiendotoxin monoclonal antibodies on endotoxin-induced lung injury in the rat. *Am. Rev. Resp. Dis.* 135:665.
- Tune, B.M., C.Y. Hsu, M. Bieber, and N.N.H. Teng. 1989. Effects of anti-lipid A human monoclonal antibody on lipopolysaccharide-induced toxicity to the kidney. *J. Urol.* 141:1463.
- Wheeler, A.P., W.D. Hardie, and G. Bernard. 1990. Studies of an antiendotoxin antibody in preventing the physiologic changes of endotoxemia in awake sheep. *Am. Rev. Resp. Dis.* 142:775.
- Young, L.S., R. Gascon, S. Alam, and L.E.M. Bermudez. 1989. Monoclonal antibodies for treatment of gram-negative infections. *Rev. Infect. Dis.* 11:S1564.
- Rudbach, J.A., F.I. Akiya, R.J. Elin, H.D. Hochstein, M.K. Luoma, E.C.B. Milner, K.C. Milner, and K.R. Thomas. 1976. Preparation and properties of a national reference endotoxin. *J. Clin. Microbiol.* 3:21.
- Warren, H.S., M. Glennon, F.A. de Deckker, and D. Tello. 1991. 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.* 163:1256.
- Westphal, O., and K. Jann. 1965. Bacterial lipopolysaccharides. *Methods Carbohydr. Chem.* 5:83.
- Romeo, D., A. Girard, and L. Rothfield. 1970. Reconstitution of a functional membrane enzyme system in a monomolecular film. I. Formation of mixed monolayer of lipopolysaccharide and phospholipid. *J. Mol. Biol.* 53:475.
- Novitsky, T.J., P.F. Roslansky, G.R. Siber, and H.S. Warren. 1985. A turbidometric method for quantifying serum inhibition of limulus amoebocyte lysate response. *J. Clin. Microbiol.* 20:211.
- Galanos, C., O. Luderitz, and O. Westphal. 1969. A new method for the extraction of R lipopolysaccharides. *Eur. J. Biochem.* 9:245.
- Farr, R.S. 1958. A quantitative immunochemical measure of

- the primary interaction between I* BSA and antibody. *J. Infect. Dis.* 103:239.
35. Warren, H.S., T.J. Novitsky, A. Bucklin, S.A. Kania, and G.R. Siber. 1987. Endotoxin neutralization with rabbit antisera to *Escherichia coli* J5 and other gram-negative bacteria. *Infect. Immun.* 55:1668.
 36. Jacobs, D.M., and D.C. Morrison. 1975. Dissociation between mitogenicity and immunogenicity of TNP-lipopolysaccharide, a T-independent antigen. *J. Exp. Med.* 141:1453.
 37. Munoz, C., J. Carlet, C. Fitting, B. Misset, J.P. Bleriot, and J.-M. Cavaillon. 1991. Dysregulation of in vitro cytokine production by monocytes during sepsis. *J. Clin. Invest.* 88:1747.
 38. Tucker, S.B., R.V. Pierre, and R.E. Jordon. 1977. Rapid identification of monocytes in a mixed mononuclear cell preparation. *J. Immunol. Methods.* 14:267.
 39. Wilson, B.M.G., A. Severn, N.T. Rapson, J. Chana, and P. Hopkins. 1991. A convenient human whole blood culture system for studying the regulation of tumour necrosis factor release by bacterial lipopolysaccharide. *J. Immunol. Methods.* 139:233.
 40. Van Snick, J., S. Cayphas, A. Vink, C. Uyttenhove, P.G. Coulie, M.R. Rubira, and R.J. Simpson. 1986. Purification and NH₂-terminal amino acid sequence of a T-cell-derived lymphokine with growth factor activity for B-cell growth/cell kill. *Proc. Natl. Acad. Sci. USA.* 83:9679.
 41. Gazzano-Santoro, H., J.B. Parent, D.M. Wood, E. Lim, P.T. Pruyne, P.W. Trown, and P.J. Conlon. 1991. Reactivity of E5 monoclonal antibody to smooth lipopolysaccharides. Program and Abstracts of the 31st Intersci. Conf. Antimicrob. Agents Chemother. pg. 230.
 42. Bogard, W.C., Jr., and S.A. Siegel. 1991. The human monoclonal antibody HA-1A: studies on the epitope location within the endotoxin molecule and epitopic exposure on the surface of viable gram-negative bacteria. *Circ. Shock.* 34:119. (Abstr.)
 43. Baumgartner, J.-D., D. Heumann, and M.-P. Glauser. 1991. The HA-1A monoclonal antibody for gram-negative sepsis. *N. Engl. J. Med.* 325:281.
 44. Galanos, C., O. Luderitz, E.T. Rietschel, O. Westphal, H. Brade, L. Brade, M.A. Freudenberg, U.F. Schade, M. Imoto, H. Yoshimura, et al. 1985. Synthetic and natural *Escherichia coli* free lipid A express identical endotoxic activities. *Eur. J. Biochem.* 148:1.
 45. Okusawa, S., J.A. Gelfand, T. Ikejima, R.J. Connolly, and C.A. Dinarello. 1988. Interleukin 1 induces a shock-like state in rabbits. *J. Clin. Invest.* 81:1162.
 46. Michie, H.R., K.R. Manogue, D.R. Spriggs, A. Revhaug, S. O'Dwyer, C.A. Dinarello, A. Cerami, S.M. Wolff, and D.W. Wilmore. 1988. Detection of circulating tumor necrosis factor after endotoxin administration. *N. Engl. J. Med.* 318:1481.
 47. Cannon, J.G., R.G. Tompkins, J.A. Gelfand, H.R. Michie, G.G. Stanford, J.W.M. Van der Meer, S. Endres, G. Lonnemann, J. Corsetti, B. Chernow, et al. 1990. Circulating interleukin-1 and tumor necrosis factor in septic shock and experimental endotoxin fever. *J. Infect. Dis.* 161:79.
 48. Wakabayashi, G., J.A. Gelfand, J.F. Burke, R.C. Thompson, and C.A. Dinarello. 1991. A specific receptor antagonist for interleukin 1 prevents *Escherichia coli*-induced shock in rabbits. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 5:338.
 49. Mathison, J.C., E. Wolfson, and R.J. Ulevitch. 1988. Participation of tumor necrosis factor in the mediation of gram negative bacterial lipopolysaccharide-induced injury in rabbits. *J. Clin. Invest.* 81:1925.
 50. Tracey, K.J., Y. Fong, D.G. Hesse, K.R. Manogue, A.T. Lee, G.C. Kuo, S.F. Lowry, and A. Cerami. 1987. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature (Lond.)* 330:662.
 51. Girardin, E., G.E. Grau, J.-M. Dayer, P. Roux-Lombard, The J5 Study Group, and P.-H. Lambert. 1988. Tumor necrosis factor and interleukin-1 in the serum of children with severe infectious purpura. *N. Engl. J. Med.* 319:397.
 52. Munoz, C., B. Misset, C. Fitting, J.P. Bleriot, J. Carlet, and J.-M. Cavaillon. 1991. Dissociation between plasma and monocyte-associated cytokines during sepsis. *Eur. J. Immunol.* 21:2177.
 53. Waage, A., P. Brandtzaeg, A. Halstensen, P. Kierulf, and T. Espevik. 1989. The complex pattern of cytokines in serum from patients with meningococcal septic shock. Association between interleukin 6, interleukin 1, and fatal outcome. *J. Exp. Med.* 169:333.
 54. Hack, C.E., E.R. De Groot, R.J.F. Felt-Bersma, J.H. Nuijens, R.J.M.S. Van Schijndel, A.J.M. Eerenberg-Belmer, L.G. Thijs, and L.A. Aarden. 1989. Increased plasma levels of interleukin-6 in sepsis. *Blood.* 74:1704.