Anti-Endotoxin Monoclonal Antibodies Inhibit Secretion of Tumor Necrosis Factor-α by Two Distinct Mechanisms

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Objective

To determine whether monoclonal antibodies (mAbs) directed against lipopolysaccharide (LPS, endotoxin) act by promoting LPS neutralization, LPS uptake by macrophages, or both processes, the authors assessed the effects of these agents on LPS-induced cytokine secretion and cellular uptake of LPS.

Summary Background Data

MAbs directed against LPS have been shown to attenuate LPS-induced macrophage tumor necrosis factor-α (TNF-α) secretion, a process that may contribute to protective capacity. The mechanisms by which this process occurs have not been established.

Methods

MAbs directed against LPS were evaluated in vitro for their capacity to (1) inhibit TNF-α secretion, and (2) alter fluorescein isothiocyanate-labeled LPS uptake (employing flow cytometry analysis and fluorescence microscopy) by the macrophage-like cell line RAW 264.7.

Results

MAb 8G9, an IgG$_3$ directed against the O-antigen polysaccharide region of Escherichia coli 0111:B4 LPS, significantly reduced LPS-induced TNF-α secretion and promoted a more than 40-fold increase in LPS uptake by macrophages. The authors established that this was mediated by a Fc receptor-mediated process because 8G9 F(ab')$_2$ fragments that lack the Fc portion of the IgG molecule were capable of inhibiting TNF-α secretion, but did not promote increased LPS uptake to the same degree. Cross-reactive, anti-deep core/lipid A mAb 1B6, an IgG$_{2a}$, also promoted uptake of E. coli 0111:B4 LPS and O-antigen polysaccharide-deficient E. coli J5 LPS, but only inhibited TNF-α secretion induced by E. coli J5 LPS to which it binds most efficiently. MAb 3D10, an IgM also directed against the O-antigen polysaccharide region of E. coli 0111:B4 LPS, inhibited TNF-α secretion but did not increase cellular uptake of LPS, presumably acting solely due to LPS neutralization. Polymyxin B, an antibiotic that binds stoichiometrically to the lipid A portion of LPS, inhibited TNF-α secretion and prevented cellular LPS uptake.

Conclusions

These results suggest that IgG and IgM anti-LPS mAbs exert protective capacity by extracellular neutralization of LPS, while IgG Fc receptor-mediated cellular uptake also may serve to bypass macrophage activation and TNF-α secretion by promoting internalization and intracellular neutralization.

Despite optimal use of standard treatment modalities that include administration of antibiotics, fluid resuscitation, hemodynamic monitoring, and nutritional support, gram-negative bacterial sepsis remains a significant cause of morbidity and mortality in hospitalized patients.[1-3] It has now been established that many if not all of the pathophysiologic sequelae observed during serious gram-negative bacterial infections (fever, hypotension, metabolic acidosis, shock, multiple organ failure, and death) are related to the effects of endotoxin (lipopolysaccharide [LPS]), an integral component of the outer membrane of gram-negative bacteria, upon the mammalian host.[4,5] LPS provokes the release of a series of so-called secondary mediators that appear to be responsible for these deleterious effects. Although many mediators may contribute to this process, LPS is capable of causing the sequential release of large amounts of cytokines (tumor necrosis factor-α [TNF-α], interleukin-1 [IL-1], interleukin-6 [IL-6], and interleukin-8 [IL-8]) from macrophages. Secretion of TNF-α, in particular, is associated with the occurrence of lethal sequelae during experimental and clinical gram-negative bacterial sepsis.

In an attempt to attenuate the toxic effects of LPS during gram-negative bacterial infection, the effects of administration of polyclonal or monoclonal antibody (mAb) preparations that are directed against LPS have been assessed in a large number of animal studies and, more recently, in large-scale clinical trials.[1,3,6-8] MAbs have been developed against the three principal regions of the LPS molecule that comprise (1) the outermost O-antigen polysaccharide region that is unique to each particular strain of gram-negative bacteria, and (2) the core polysaccharide region that is similar among many strains of gram-negative bacteria that couples O-antigen polysaccharide to (3) the highly conserved lipid A region that represents that portion of the LPS molecule responsi-

ble for toxicity. Because the protective capacity of antibody directed against the antigenically diverse O-antigen polysaccharide region of LPS is limited to infection caused by a specific organism, attention has focused on the development and assessment of cross-reactive mAbs that bind to common antigens on LPS derived from many different gram-negative bacterial genera and species.[6] MAbs directed against the antigenically conserved deep core/lipid A (DCLA) region of LPS are among a growing list of agents that include lipid A analogs, lipopolysaccharide binding protein (LBP), bactericidal/permeability increasing protein (BPI), and polymyxin B, which block the interaction of lipid A with host cells and which have been reported to attenuate the biologic effects of LPS.[9-15]

Despite the favorable results obtained in experimental studies employing anti-LPS mAbs *in vitro* and *in vivo*, critical questions remain regarding the precise mechanisms by which exogenously administered anti-LPS mAbs or endogenous anti-LPS antibodies may serve to attenuate the toxicity of LPS and provide protective capacity during gram-negative bacterial infection. This issue has become of considerable importance based upon the controversy that has arisen concerning the results of clinical trials of anti-LPS mAbs during the treatment of sepsis. These trials indicate that the mAb agents tested (HA-1A and E5) demonstrate at most modest efficacy, but the suspicion exists that these reagents may not effectively bind and neutralize the effects of LPS.[1,3,7,8]

It has been proposed that anti-LPS mAbs may act by (1) enhancing microbial killing, thereby preventing bacterial proliferation and endotoxin release; (2) neutralizing the toxic effects of LPS; (3) promoting LPS clearance from the systemic circulation; or (4) a combination of these mechanisms.[1,12-14] Although the mechanisms by which these agents act have not been defined, anti-LPS mAbs that bind to either the non-toxic O-antigen polysaccharide region or the toxic DCLA region have been shown to inhibit the release of potentially deleterious endogenous secondary mediators *in vitro* and *in vivo* and to provide protection during gram-negative bacterial infection or endotoxemia. Therefore, particular attention has been directed at assessing the capacity of anti-LPS mAbs to attenuate the release of TNF-α. Our group and others have demonstrated that anti-LPS mAbs can attenuate LPS-induced TNF-α release by macrophages *in vi-*

tro and can reduce serum TNF-α levels and decrease mortality during experimental gram-negative bacterial sepsis or endotoxemia.[15-25]

In the current studies, we sought to examine the mechanisms by which several anti-LPS mAbs and polymyxin B influenced the interaction of LPS with the macrophage cell membrane *in vitro*. Two mAbs (8G9 [IgG$_3$] and 3D10 [IgM]) directed specifically against the O-antigen polysaccharide region of *Escherichia coli* 0111:B4 and 1B6 (IgG$_{2a}$), a cross-reactive mAb directed against the DCLA region of LPS, were examined. F(ab')$_2$ fragments that lack the Fc portion of the IgG molecule also were prepared to study the impact of Fc receptor-mediated effects. Polymyxin B, an antibiotic that binds stoichiometrically to the lipid A portion of LPS, was used as a control in all assays. Two types of LPS were examined: (1) *E. coli* 0111:B4 LPS that possesses a unique O-antigen polysaccharide region, complete core LPS, plus lipid A; and (2) *E. coli* J5 LPS that lacks O-antigen polysaccharide and expresses only a portion of core LPS plus lipid A. First, we sought to determine the effect of these various agents upon LPS-induced TNF-α secretion *in vitro*. Next, using anti-LPS mAbs as probes, we sought to examine the mechanism by which TNF-α secretion might be inhibited by determining the effects of these mAbs on the interaction of fluorescein isothiocyanate-labeled LPS (FITC-LPS) with the macrophage cell membrane, using both flow cytometry and fluorescence microscopy. We hypothesized that different classes of anti-LPS mAbs might act by different mechanisms, neutralizing LPS, enhancing cellular uptake of bound LPS, or both.

MATERIALS AND METHODS

LPS Preparations

LPS derived from *E. coli* 0111:B4 and its UDP-galactose epimerase deficient mutant *E. coli* J5 were obtained from List Biological Laboratories (Campbell, CA). FITC-*E. coli* 0111:B4 was obtained from Sigma Chemical Co. (St. Louis, MO). FITC-*E. coli* J5 was prepared using modifications of a procedure described by Skelley et al.[26] Briefly, 5 mg of *E. coli* J5 LPS was incubated with 10 mg of FITC in 2.5 mL of 0.1 M sodium carbonate (pH 9.5) for 2 hours on a rotary shaker at 37 C. The mixture was then dialyzed against phosphate-buffered saline (PBS) for 5 days. The amount of FITC association with LPS was determined using the thiobarbituric acid assay.[27] The FITC contents of FITC-*E. coli* 0111:B4 and FITC-*E. coli* J5 were determined spectrophotometrically at 493 nm using an extinction coefficient of 85,200 M^{-1} cm^{-1}; the values were 10 μg/mg and 7.5 μg/mg, respectively.

Production and Characterization of Anti-LPS MAbs

The development and characterization of anti-LPS mAbs 8G9, 3D10, and 1B6 have been previously described.[23] Briefly, mAb 8G9, an IgG$_3$, and mAb 3D10, an IgM, were produced by immunizing Balb/c mice with *E. coli* 0111:B4 LPS; each binds exclusively to the O-antigen polysaccharide region of *E. coli* 0111:B4 LPS. MAb 1B6, an IgG$_3$, was produced by immunizing mice with live and heat-killed *E. coli* J5 and *Salmonella minnesota* Re and their derived LPS. MAb 1B6 recognizes an epitope within the DCLA region of LPS and demonstrates cross-genera binding by Western immunotransblot analysis, immunodotblot analysis, and enzyme-linked immunosorbent assay (ELISA). MAb 8D11, an IgG$_3$, was produced by immunization with *S. minnesota* wild type LPS and is directed against the O-antigen polysaccharide region of this type of LPS. It does not demonstrate binding to either *E. coli* 0111:B4 or *E. coli* J5 by Western immunotransblot analysis, immunodotblot analysis, or ELISA. Each mAb preparation was produced from murine ascites, purified by affinity chromatography, and extensively dialyzed against PBS before use. IgG mAbs were purified by passage over a protein A column, while IgM mAbs were purified using a diethylaminoethyl (DEAE) disk employing a technique that we described previously.[28] MAb preparations that were used in these experiments exhibited an endotoxin content of less than 0.1 ng/mL, as determined by the Limulus amebocyte lysate assay.[29]

Preparation and Characterization of Anti-LPS MAb F(ab')$_2$ Fragments

F(ab')$_2$ fragments derived from anti-LPS mAbs 8G9 and 1B6 were prepared using a procedure described by Lamoyi.[30] Ten milliliters of a 2 mg/mL solution of each anti-LPS mAb was dialyzed against 0.1 M sodium acetate buffer overnight at 4 C. Immediately before digestion, the antibody solution was pH adjusted to pH 4.5 for mAb 8G9 and pH 4.2 for mAb 1B6 using 2 M acetic acid. A 25 mg/mL solution of pepsin (Sigma Chemical Co.) was prepared in sodium acetate buffer. The mAb and pepsin solutions were warmed to 37 C, and pepsin was added to the antibody solution to produce an enzyme:mAb weight ratio of 1:33. Optimal digestion times were determined in preliminary studies (15 minutes for mAb 8G9 and 12 hours for mAb 1B6). Digestion was halted by adding a 1:40 volume of 2 M Tris-HCl (pH 8.0) and adjusting the pH to 8.0 using 1 N NaOH. Undigested whole molecules and Fc fragments were removed by passing the digested solution over a protein A column. The solution containing F(ab')$_2$ mAb fragments was extensively dialyzed against PBS and the endotoxin

content of the F(ab')$_2$ mAb fragments was determined. The purity of the F(ab')$_2$ solution was confirmed by sodium dodecyl polyacrylamide gel electrophoresis analysis under reduced conditions using 15% gels. The affinity of intact mAb and F(ab')$_2$ mAb fragments was assessed by ELISA using an ammonium thiocyanate elution technique.[31,32] The resulting F(ab')$_2$ fragment preparations were found to be free of IgG whole molecule contamination, contained less than 0.1 ng/mL of contaminating endotoxin, and demonstrated binding affinity similar to the intact IgG mAbs from which they were derived (data not shown).

Measurement of Secretion of TNF-α by LPS-Stimulated RAW 264.7 Cells

RAW 264.7 cells (5 × 10^5) were incubated for 16 hours in 1 mL of Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) at 37 C in a 10% CO$_2$ atmosphere. On the following day, supernatants were removed and 1 mL of serum-free DMEM or 1 mL of serum-free DMEM containing either LPS (100 ng/mL), mAb (100 μg/mL), or both LPS and mAb that had been preincubated for 1 hour at room temperature was added to each well. The concentration of LPS used had been selected to achieve near maximal TNF-α secretion in this assay system (data not shown). Polymyxin B (10 μg/mL) also was used in place of anti-LPS mAbs in each series of experiments. After incubation for 3 hours at 37 C, supernatants were removed and stored at -80 C.

ELISA was used to determine the amount of TNF-α in supernatant samples. Fifty microliters of 2 μg/mL anti–TNF-α mAb (mAb MP6-XT3, Pharmingen, San Diego, CA) diluted in 0.1 M NaHCO$_3$, pH 8.2, was placed into each well of a high affinity, polystyrene microtiter plate (96-well Easy Wash plates, Corning Inc., Corning, NY). After each incubation, the plate was washed five times with PBS containing 0.05% Tween 20. After incubation at 4 C overnight, unbound sites on the plate were blocked by adding 200 μL of 20% FCS diluted in PBS and plates were incubated at room temperature for 2 hours. Samples to be assayed (100 μL of diluted supernatant in PBS containing 10% FCS) were added to each well and allowed to incubate at room temperature for 1 hour. One hundred microliters of 1 μg/mL biotinylated anti–TNF-α mAb (mAb MP6-XT22, Pharmingen) diluted in PBS containing 10% FCS was then incubated in each well at room temperature for 30 minutes. Next, 100 μL of a 2.5 μg/mL solution of horseradish peroxidase-labeled avidin D (Sigma Chemical Co.) diluted in PBS containing 10% FCS was incubated in each well at room temperature for 30 minutes. One hundred microliters of substrate (5.5 × 10^{-4} M 2,2'-azino-bis[3-ethylbenzthia-

zoline-6-sulfonic acid] in 0.1 M citric acid plus 0.03% H$_2$O$_2$) was added and the absorbance at 405 nm was determined. The amount of TNF-α in each sample was calculated from a standard curve generated using recombinant murine TNF-α (Genzyme Corp., Cambridge, MA).

Analysis of FITC-LPS Uptake by RAW 264.7 Cells Using Flow Cytometry Analysis

Anti-LPS mAb (100 μg/mL) or polymyxin B (10 μg/mL) was combined with FITC-LPS (1 μg/mL) in serum-free DMEM and incubated on a rotary shaker for 1 hour at room temperature. The concentration of FITC-LPS used was found to achieve an optimal fluorescence signal in this assay system. RAW 264.7 cells (10^6) were resuspended in 1 mL of mAb-LPS mixture in 12 × 75-mm polystyrene tubes and incubated for 3 hours in a 10% CO$_2$ atmosphere at 37 C. Cells were washed three times by centrifugation (10 minutes × 1000 rpm) and then resuspended in PBS at 4 C. After the third wash, the cell pellet was resuspended in PBS containing 2% formaldehyde and was stored at 4 C. To distinguish cell surface associated LPS from internalized FITC-LPS, trypan blue was added. This dye is excluded from viable cells and quenches extracellular fluorescence.[33,34] In these assays, cells were incubated with 0.02% trypan blue in sodium acetate buffer (pH 5.8) for 15 seconds at room temperature after the first cell wash, and cells were further processed as previously described.

FITC-LPS binding to RAW 264.7 cells was evaluated using a FACScan (Becton Dickinson, San Jose, CA) analyzer that was equipped with an argon ion laser operating at 488 nm with a 60-mW light source. The fluorescence signal was measured through a filter (530/30 nm). Fluorescence intensity was recorded on a logarithmic scale in relative fluorescence units (FU) and was analyzed using the Consort-30 software program (Becton Dickinson). Side scatter parameters were used to apply computerized gating to eliminate nonviable cells and debris from analysis, and the mean fluorescence intensity of 10,000 cells was determined for each sample. Substitution of free FITC combined with RAW 264.7 cells in place of FITC-LPS did not produce cellular fluorescence.

Analysis of FITC-LPS Uptake by RAW 264.7 Cells Using Fluorescence Microscopy

FITC-LPS binding to RAW 264.7 cells also was examined by fluorescence microscopy. Before formalin fixation, cells were fixed to the surface of glass microscope plates using a cytospin technique. Fluorescence was assessed by direct visualization using an Olympus BH2

microscope (Olympus Optical, Tokyo, Japan) equipped with epifluorescence. Light microscopic images were photographed using transmitted light at 40X and 100X magnification. Images were recorded on Kodak Ektachrome 400 ASA color slide film (Eastman Kodak Co., Rochester, NY). At least three separate fields were recorded per coverslip.

Statistical Analysis

Each assay was performed at least three times. Results were compared using the unpaired Student's t test. All results were expressed as the mean ± standard deviation. Per cent inhibition was calculated for the TNF-α assay results using the equation:

$$\% \text{ inhibition} = \frac{(X_{LPS} - X_{Medium}) - (X_{LPS + mAb} - X_{mAb})}{(X_{LPS} - X_{Medium})}$$

where X_y equals the value obtained in each assay using LPS alone, medium alone, mAb alone, or LPS plus mAb.

RESULTS

Effects of Anti-LPS MAbs on LPS-Stimulated TNF-α Secretion by RAW 264.7 Cells

Anti-*E. coli* 0111:B4 mAb 8G9, mAb 8G9 F(ab')$_2$ fragments, and anti-*E. coli* 0111:B4 mAb 3D10 significantly reduced the amount of TNF-α secreted by RAW 264.7 cells in response to *E. coli* 0111:B4 LPS, compared to either control mAb 8D11 or medium alone (p < 0.001) (Fig. 1). MAb 8G9, mAb 8G9 F(ab')$_2$ fragments, and mAb 3D10 had no effect on *E. coli* J5 LPS-induced TNF-α secretion. Anti-deep core/lipid A mAb 1B6 significantly reduced TNF-α secretion stimulated by *E. coli* J5 LPS (p < 0.01), but not *E. coli* 0111:B4 LPS, compared to control mAb 8D11 or medium alone. MAb 1B6 F(ab')$_2$ fragments had no effect on either *E. coli* 0111:B4 or *E. coli* J5 LPS-induced TNF-α secretion. Polymyxin B significantly decreased TNF-α secretion in response to both *E. coli* 0111:B4 and *E. coli* J5 LPS, compared to control mAb or medium alone (p < 0.001) (Fig. 1).

Flow Cytometry Analysis of FITC-LPS Uptake by RAW 264.7 Cells

Unless otherwise indicated in these studies, the term "uptake" will refer to any increase in FITC-LPS association with cells, whether due to an increase in cell membrane attachment or internalization of FITC-LPS. Incubation of mAb 8G9 resulted in a more than 40-fold uptake of FITC-*E. coli* 0111:B4 LPS with RAW 264.7 cells, compared to either control mAb 8D11 or medium alone (p < 0.001) (Fig. 2 and Table 1). Incubation of FITC-*E.*
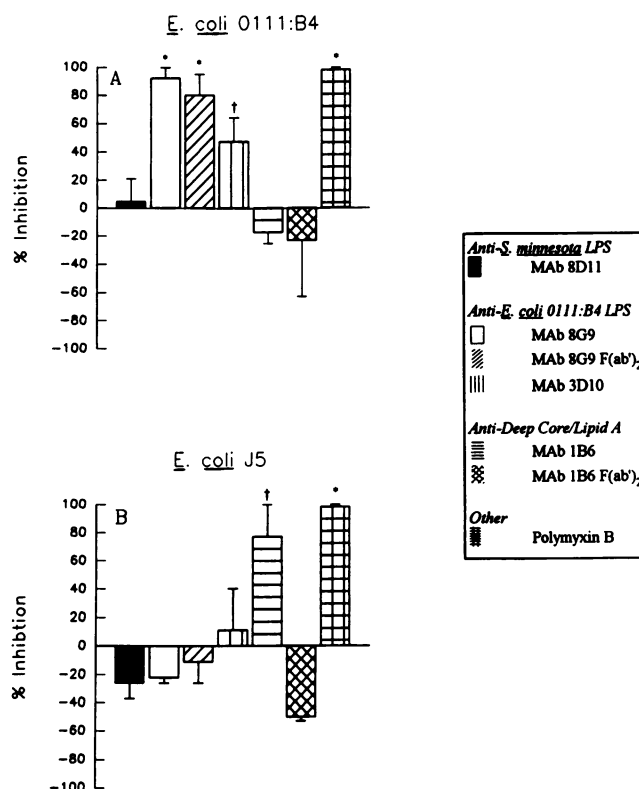

Figure 1. Effect of anti-LPS mAbs and polymyxin B on TNF-α secretion by LPS-stimulated RAW 264.7 cells. (A) *E. coli* 0111:B4 or (B) *E. coli* J5 LPS was premixed with anti-LPS mAbs or polymyxin B and then incubated with 5 × 10^5 RAW 264.7 cells. Supernatants were collected after 3 hours and assayed for TNF-α levels. *p < 0.001 and †p < 0.01, compared to control mAb.

coli 0111:B4 LPS with varying amounts of mAb 8G9 demonstrated that as little as 10 μg/mL of mAb resulted in significant LPS uptake (Fig. 3). Trypan blue quenching of cell membrane associated FITC revealed that a greater percentage of FITC-LPS was internalized after incubation with mAb 8G9, compared to incubation without mAb (58 ± 3% vs. 39 ± 5%; p < 0.01; Table 2). In contrast to intact IgG mAb 8G9, mAb 8G9 F(ab')$_2$ fragments did not enhance uptake of FITC-LPS with macrophages to the same degree. Also, in contradistinction to IgG$_3$ mAb 8G9, IgM mAb 3D10 had no effect on FITC-*E. coli* 0111:B4 uptake by macrophages compared to control mAb 8D11 or medium alone. Polymyxin B decreased both FITC-*E. coli* 0111:B4 and FITC-*E. coli* J5 LPS uptake by macrophages, compared to medium alone (Fig. 2 and Table 1).

To determine whether the process of Fc receptor-mediated uptake of LPS also occurred with mAbs recognizing epitopes within the DCLA region of LPS, experiments were performed using IgG$_{2a}$ mAb 1B6 that exhibits anti-DCLA specificity and F(ab')$_2$ fragments derived from this mAb. Incubation of mAb 1B6 resulted in a more than twofold increase in uptake of either
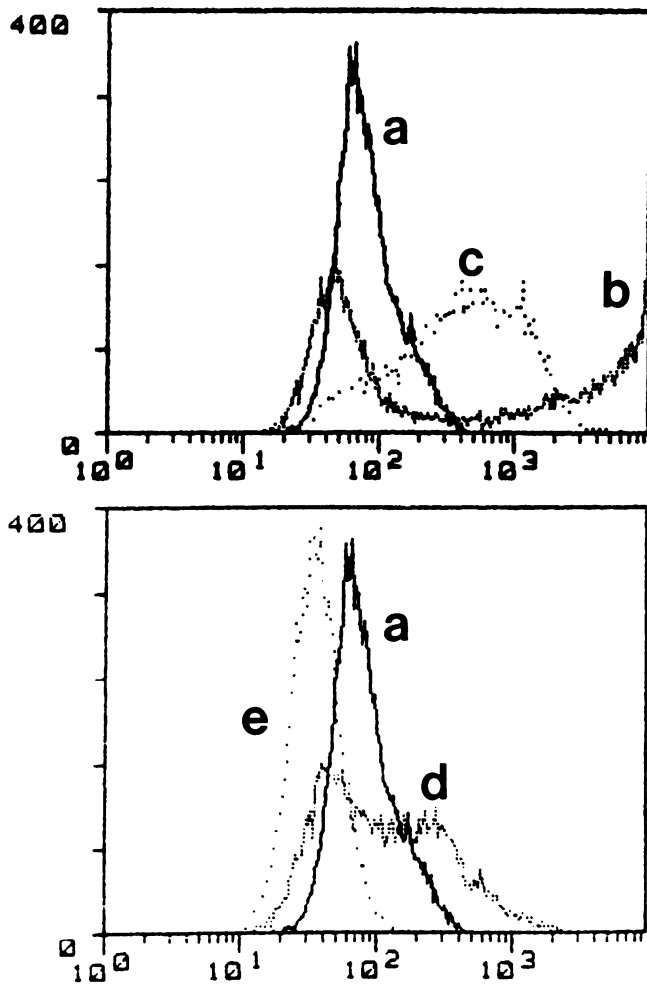
Figure 2. Effect of anti-LPS mAbs or polymyxin B on FITC-*E. coli* 0111:B4 LPS association with RAW 264.7 cells, as analyzed by flow cytometry. RAW 264.7 cells (10⁶) were incubated for 3 hours at 37 C with 1 μg/mL of FITC-*E. coli* 0111:B4 LPS with 100 μg/mL of (a) mAb IgG₃ 8D11 control mAb, (b) IgG₃ anti-*E. coli* 0111:B4 mAb 8G9, (c) 8G9 mAb F(ab')₂ fragments, (d) IgM anti-*E. coli* 0111:B4 mAb 3D10, or (e) polymyxin B. X axis represents fluorescence intensity (relative units). Y axis represents number of cells.

FITC-*E. coli* 0111:B4 or FITC-*E. coli* J5 LPS with RAW 264.7 cells, compared to control mAb or medium alone. In contrast, 1B6 F(ab')₂ fragments did not significantly lead to uptake of FITC-LPS by macrophages compared to control mAb or medium alone using either *E. coli* 0111:B4 or *E. coli* J5 LPS (Table 1).

Fluorescence Microscopic Analysis of FITC-*E. coli* 0111:B4 LPS Uptake by RAW 264.7 Cells

To assess the manner in which anti-LPS mAbs influenced the interaction of FITC-*E. coli* 0111:B4 LPS with RAW 264.7 cells, samples were analyzed using fluorescence microscopy. Incubation with control mAb

8D11 or medium alone resulted in no observable FITC-*E. coli* 0111:B4 LPS uptake by RAW 264.7 cells. In contrast, incubation of FITC-LPS with mAB 8G9 produced a marked uptake of FITC-*E. coli* 0111:B4 LPS into discrete, cytoplasmic vacuoles compatible with phagocytosis of mAb-LPS complexes. No observable alteration in FITC-LPS uptake was seen after incubation of FITC-LPS with mAb 8G9 F(ab')₂ fragments, mAb 3D10, or polymyxin B (data not shown).

DISCUSSION

During severe gram-negative bacterial infection, proliferating and disrupted gram-negative bacterial organisms release endotoxin (LPS), a process that triggers the activation of the host immune response, the release of acute phase reactants (fibrinogen, C-reactive protein, and haptoglobin) by hepatic parenchymal cells, and the production of cytokines. Over the last decade, it has become increasingly evident that this host response to gram-negative bacterial infection is highly dependent upon effector cell recognition of LPS. Recognition of LPS by leukocytes is a complex process that involves both cell membrane receptors and interactions with components of serum. Several putative LPS membrane receptors that may be critical to the induction of cellular responses to LPS have been identified on leukocytes and other types of cells. These receptors include (1) molecules of the CD11/CD18 (leukocyte integrin) group that bind LPS present on intact gram-negative bacteria or
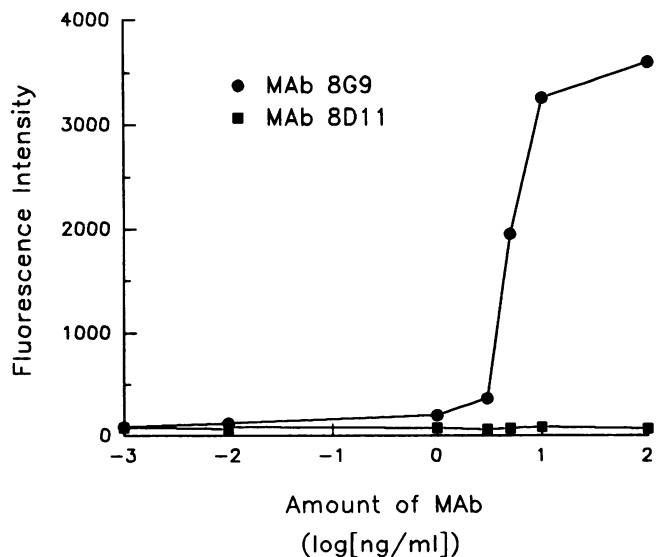

Figure 3. Effect of amount of anti-LPS mAb 8G9 on FITC-*E. coli* 0111:B4 LPS uptake by RAW 264.7 cells. RAW 264.7 cells (10⁶) were incubated with FITC-*E. coli* 0111:B4 LPS with increasing amounts of anti-*E. coli* 0111:B4 mAb 8G9. FITC association with cells was assessed by flow cytometry.

Table 1. EFFECT OF ANTI-LPS mAbs AND POLYMYXIN B ON RAW 264.7 CELL UPTAKE OF FITC-*Escherichia coli* 0111:B4 OR *E. coli* J5 LPS

Type of LPS	LPS + Agent	Anti-LPS mAb Binding Specificity	Antibody Class	Fluorescence Intensity (FU)	p Value‡
E. coli 0111:B4	Medium	—	—	78.6 ± 26.4* (17)†	—
	8D11	*Salmonella minnesota*	IgG₃	68.9 ± 13 (5)	—
	8G9	*E. coli* 0111:B4	IgG₃	3584.0 ± 1074.1 (9)	< 0.001
	8G9 F(ab′)₂	*E. coli* 0111:B4	IgG₃	635.6 ± 412.5 (4)	< 0.05
	3D10	*E. coli* 0111:B4	IgM	124.1 ± 89.4 (5)	NS
	1B6	Deep core/lipid A	IgG₂ₐ	371.7 ± 146.6 (6)	< 0.05
	1B6 F(ab′)₂	Deep core/lipid A	IgG₂ₐ	94.6 ± 17.1 (4)	NS
	Polymyxin B	—	—	33.7 ± 7.8 (7)	< 0.001
E. coli J5	Medium	—	—	282.3 ± 107.1 (13)	—
	8D11	*S. minnesota*	IgG₃	200.4 ± 6.2 (3)	NS
	8G9	*E. coli* 0111:B4	IgG₃	336.5 ± 218.3 (3)	NS
	8G9 F(ab′)₂	*E. coli* 0111:B4	IgG₃	327.7 ± 60.9 (3)	NS
	3D10	*E. coli* 0111:B4	IgM	370.1 ± 140.2 (3)	NS
	1B6	Deep core/lipid A	IgG₂ₐ	806.6 ± 196.1 (3)	< 0.05
	1B6 F(ab′)₂	Deep core/lipid A	IgG₂ₐ	264.3 ± 67.2 (3)	NS
	Polymyxin B	—	—	142.8 ± 80.5 (5)	< 0.05

FU: relative fluorescence units; NS: not significant.
* Mean ± SD.
† Number.
‡ Compared to control mAb 8D11.

incorporated into liposomes; (2) CD14, a human leukocyte receptor that recognizes LPS complexed to LBP; (3) acetyl-low density lipoprotein receptors that bind to lipid A derivatives; (4) less well-identified membrane proteins that appear to bind various regions of the LPS molecule; and (5) nonspecific interactions with membrane lipids.[1,35] Identification of the precise cell surface components that bind to LPS and mediate LPS-induced cellular dysfunction will probably be critical to the development of new treatment strategies directed at attenuating the deleterious effects of LPS *in vivo*. The capacity of anti-LPS mAbs and polymyxin B to attenuate the effects of LPS both *in vitro* and *in vivo* suggests that these agents may interfere with normal cellular recognition of LPS and activation in response to an LPS stimulus. Thus, these reagents can be used as probes to dissect out the manner in which LPS provokes the host septic response.

Several studies have attempted to evaluate the effects of anti-LPS mAbs on LPS binding to macrophages. For example, Weersink et al. reported that two anti-lipid A mAbs significantly inhibited the uptake of *S. minnesota* Re LPS by human macrophages.[34] Although these authors hypothesized that these mAbs may serve to block lipid A binding to the macrophage cell membrane and thereby neutralize the activity of LPS, cytokine secretion was not assessed. Only two studies have correlated inhibition of LPS binding to macrophages with decreased cytokine secretion. In one study evaluating several anti-lipid

Table 2. EFFECT OF ANTI-LPS mAb 8G9 ON INTERNALIZATION OF FITC-*Escherichia coli* 0111:B4 LPS BY RAW 264.7 CELLS

Monoclonal Antibody	Total Fluorescence (FU)	Internalized Fluorescence (FU)	% of Fluorescence Internalized
None	77.2 ± 36.5* (4)†	29.1 ± 11.2 (4)	39 ± 5
mAb 8G9	4411.8 ± 297.7 (3)	2565.3 ± 138.7 (3)	58 ± 3‡

FU: relative fluorescence units.
* Mean ± SD.
† Number.
‡ p < 0.01 compared to cells incubated with *E. coli* 0111:B4 LPS without mAb 8G9.

A mAbs, Lasfargues et al. reported that two IgM mAbs inhibited IL-1 secretion in response to *E. coli* LPS and significantly inhibited cellular uptake of LPS.[16] Three additional IgM anti-lipid A mAbs were found to exhibit a limited capacity to inhibit TNF-α secretion (< 40% inhibition) and either did not alter or enhanced LPS uptake by macrophages. More recently, Heumann et al. reported that an IgG₁ anti-*E. coli* 0111:B4 mAb inhibited TNF-α secretion but enhanced macrophage uptake of *E. coli* 0111:B4 LPS.[25] Anti-LPS mAb uptake of LPS by macrophages was not significantly altered by anti-CD14 mAbs, suggesting that this process was independent of the interaction between LBP-LPS complexes and the CD14 receptor. These latter observations are particularly relevant considering mounting evidence that the interaction of LPS with plasma protein components (primarily LBP) markedly enhances the sensitivity of leukocytes to LPS and may be a critical step in triggering the host cytokine response during gram-negative bacterial infection.[35,36]

In this current study, we sought to determine whether anti-LPS mAbs serve to attenuate TNF-α secretion by promoting LPS neutralization, by LPS uptake, or by both mechanisms. Our study differs from previous studies in that we directly evaluated Fc receptor-mediated LPS uptake by using IgG mAbs, F(ab′)₂ fragments that lack the Fc portion of the IgG molecule that binds to the mammalian Fc cellular receptor, IgM mAbs, and polymyxin B. We sought to evaluate the effects of LPS on the macrophage membrane using two separate techniques —flow cytometry and fluorescence microscopy. Because lipid A and not the core or O-antigen polysaccharide regions of LPS has been hypothesized to be involved in the binding of LPS to macrophages, we evaluated the effects of agents that selectively bound to each respective region on LPS uptake.[37,38] To further define the effects of the O-antigen polysaccharide region on LPS binding to macrophages, we performed these experiments using both complete (*E. coli* 0111:B4) and O-antigen polysaccharide-deficient (*E. coli* J5) LPS.

Consistent with previous studies by our group, anti-*E. coli* 0111:B4 mAbs 8G9 and 3D10 were shown to significantly reduce TNF-α secretion induced by LPS possessing the O-antigen polysaccharide region to which these mAbs bind efficiently.[23] Also concordant with our previous findings, anti-DCLA mAb 1B6 was observed to attenuate TNF-α secretion in response to *E. coli* J5 LPS (an antigen used to produce this mAb), but not *E. coli* 0111:B4 LPS. In the current studies, we further demonstrated that both intact IgG molecules and F(ab′)₂ fragments were capable of attenuating TNF-α secretion, suggesting that Fc receptor-mediated interactions were not an absolute requirement for the neutralizing actions of these mAbs. Although IgG mAbs, IgG mAb F(ab′)₂ frag-

ments, and IgM mAbs were shown to have similar effects on TNF-α secretion, studies using FITC-LPS revealed that these mAbs may have achieved these effects by two distinct mechanisms. IgG mAbs markedly enhanced LPS uptake by macrophages primarily by uptake into intracellular vacuoles. Experiments using F(ab′)₂ fragments suggested that the marked uptake mediated by these mAbs was due principally to a Fc receptor-mediated process. In contrast to the marked uptake observed with intact IgG molecules, IgM molecules had no significant effect on uptake of LPS by macrophages. This finding was observed using either the sensitive technique of flow cytometry analysis or fluorescence microscopy.

Our results suggest that inhibition of LPS uptake by macrophages and Fc receptor-mediated internalization of LPS both serve to preclude the interaction of LPS with the macrophage cell membrane. Because these two processes occurred in the absence of serum components, it is unlikely that binding of LPS to LBP and the subsequent interaction of these complexes with CD14-like receptors of RAW 264.7 cells contributed to the effects of anti-LPS antibodies in this system. Direct confirmation of this hypothesis, however, will require re-evaluation of the effects of anti-LPS antibodies in an experimental system in which LPS-LBP/CD14 receptor interactions can be evaluated precisely.

These studies also suggest that the amount of IgG mAb-mediated uptake of FITC-LPS uptake may correlate with the degree to which these agents attenuate TNF-α secretion. Although anti-DCLA mAb 1B6 promoted the uptake of *E. coli* 0111:B4, the amount of LPS uptake was significantly lower than that observed with anti-O-antigen polysaccharide mAb 8G9. This observation indicates that a substantial amount of free LPS may remain in the medium unbound by mAb and was available for interaction with the cell membrane and stimulation of TNF-α secretion. These findings may be explained by the affinity of mAb 8G9 for LPS being significantly higher than that of mAb 1B6, or alternatively that the hydrophobic DCLA epitope that mAb 1B6 recognizes may be relatively concealed within LPS micelles that form in aqueous solution. Nevertheless, both anti-O-antigen polysaccharide and anti-DCLA IgG mAbs promoted LPS uptake, demonstrating that IgG mAbs that bind to either region of LPS can facilitate this process. The observation that mAb 1B6 inhibited *E. coli* J5 LPS-stimulated TNF-α secretion but did not promote LPS uptake to the same degree as mAb 8G9 suggests that factors other than mAb-mediated LPS uptake also contribute to the biologic activity of these agents. These findings are consistent with previous observations by our group that anti-LPS mAb binding to a certain region of LPS alone is not sufficient to predict the efficacy of these agents in biologic assays. We believe that small varia-

tions in epitope binding and mAb affinity may be critical determinants of the *in vitro* and *in vivo* effects of these agents as well.

Although anti-LPS mAbs serve to prevent LPS-induced cellular activation and TNF-α secretion in this *in vitro* system, it has yet to be established whether similar processes occur *in vivo* during gram-negative bacteremia and endotoxemia. We have previously shown that F(ab')$_2$ fragments of mAb 8D11, an IgG$_3$ anti-O-antigen polysaccharide mAb, are less effective than intact IgG in preventing mortality during lethal endotoxemia in mice.[32] While attenuation of LPS-induced TNF-α secretion by either intact IgG or F(ab')$_2$ fragments may in part be responsible for the protective capacity of these agents, Fc receptor-mediated LPS clearance may serve to efficiently clear bound LPS, thus preventing ongoing cellular activation by free LPS. In addition to anti-LPS mAbs, other agents that bind the toxic lipid A moiety of LPS, such as polymyxin B, BPI, or LBP, also may serve to inhibit the normal interaction of LPS with macrophages. Our current studies suggest, however, that binding to one of several regions of LPS rather than a specific requirement for binding to the toxic lipid A region is sufficient to attenuate the effect of LPS *in vitro*.

We hypothesize that abrogation of LPS-mediated toxicity can occur by either direct neutralization of LPS or by circumventing the interaction of LPS with the mammalian cell membrane. Further elucidation of both mechanisms may be critical to devising optimal forms of adjunctive therapy for gram-negative bacterial sepsis and shock.

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Discussion

DR. RICHARD L. SIMMONS (Pittsburgh, Pennsylvania): This is a typically elegant paper by Dr. Dunn and his group. He quite clearly showed that antibody to the core lipid A portion of the lipopolysaccharide (LPS) molecule, that is the business part of the molecule, the part that makes us sick, will directly neutralize the toxic component. He has further shown us that even if the antibody doesn't bind the lipid A portion (and antibodies to O polysaccharide don't seem to get there), there is another mechanism by which the cell can ingest the toxic molecule and eliminate it from its toxic mission.

Dr. Dunn and others have repeatedly shown us that antibodies to lipid A and other components of the endotoxin molecule will work to prevent LPS toxicity *in vitro*. Such antibodies do not seem to work anywhere near as well *in vivo*. So I have several questions for Dr. Dunn.

Do you have evidence that, *in vivo*, these antibodies are subject to the same mechanisms which work *in vitro*?

Second, is the RAW cell, this immortalized line of macrophages that you've used as an indicator cell, reflective of the normal macrophage *in vivo*?

And most importantly, does this antibody interact with LPS in the context of LPS presentation *in vivo*? Normally, LPS is rather avidly bound immediately in serum by lipopolysaccharide binding protein (LBP). LBP not only binds LPS, but also intensifies its action about a hundred to a thousand times. Thus, most macrophages don't ever see LPS in the form used in this assay system; instead, they see the LBP-LPS complex, which is a hundred to a thousand times more toxic. This is the reason antibodies don't work *in vivo* as well as we would hope because the antibodies in fact don't get to the lipid A when the lipid A is bound to the LBP complex.

Congratulations to Dr. Dunn and his group.

DR. BASIL A. PRUITT, JR. (Fort Sam Houston, Texas): President MacLean, Dr. Sheldon, Dr. Barker, Fellows and Guests, Dr. Dunn and his colleagues have presented the results of a study that gives promise of both defining the mechanism of protection provided by antiendotoxin antibodies and identifying a means of screening such antibodies for clinical use.

They have found that monoclonal antibodies against lipopolysaccharide (LPS) can attenuate LPS-induced macrophage tumor necrosis factor (TNF) secretion by two apparently distinct mechanisms; that is, direct LPS neutralization and Fc receptor mediated internalization that bypasses macrophage membrane interaction and influences the distribution of fluorescein-labeled LPS. They have also shown that although a monoclonal antibody against the *Escherichia coli* J5 mutant promoted macrophage uptake of the LPS produced by other *E. coli* organisms, it did not inhibit TNF secretion induced by the other LPSs. That finding may explain in part the clinical failure of HA-1A.

I have a few concerns that I ask the essayist to address to help us interpret his findings.

The fluorescein labeling of the *E. coli* J5 LPS entailed incubation, alkalization to a pH of 9.5, and dialysis. Could that processing have altered the J5 LPS and influenced its subsequent processing by macrophages or alternatively permitted elution of the fluorecein tag into the test system medium? It is noted in the manuscript that free fluorescein isothiocyanate (FITC) did not produce cellular fluorescence.

Since there are probably many LPS epitopes per LPS molecule, as the monoclonal antibody concentration is varied in a test system, there should be both soluble and insoluble complex zones. If only soluble complexes can get into cells, then any of the monoclonal antibodies could cause both intracellular and extracellular neutralization with the proportion of each being dose dependent. Have you defined the solubility zones of the several antibodies you developed and have you related internalization to those zones?

Since the antibody response to LPS is isotype restricted primarily to IgG1 and IgG2 in humans and the IgG antibodies you examined were of the IgG3 subclass, and since IgG antibodies of different subclasses exhibit different capacities to bind Fc receptors even when the different subclasses have identical variable regions, does the greater internalization of the IgG3 antibody simply reflect low avidity and/or dissolution of initial LPS binding? In fact, does the differential and species specificity of binding mean that only IgG1, IgG2, or IgM monoclonal antibodies should be candidates for clinical trials?