Lipopolysaccharide Binding Protein Enhances the Responsiveness of Alveolar Macrophages to Bacterial Lipopolysaccharide

Implications for Cytokine Production in Normal and Injured Lungs

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Abstract

A plasma lipopolysaccharide (LPS)-binding protein (LBP) has been shown to regulate the response of rabbit peritoneal macrophages and human blood monocytes to endotoxin (LPS). We investigated whether LBP is present in lung fluids and the effects of LBP on the response of lung macrophages to LPS. Immunoreactive LBP was detectable in the lavage fluids of patients with the adult respiratory distress syndrome by immunoprecipitation followed by Western blotting, and also by specific immunoassay. In rabbits, the LBP appeared to originate outside of the lungs, inasmuch as mRNA transcripts for LBP were identified in total cellular RNA from liver, but not from lung homogenates or alveolar macrophages. Purified LBP enhanced the response of human and rabbit alveolar macrophages to both smooth form LPS (*Escherichia coli* O111B:4) and rough form LPS (*Salmonella minnesota* Re595). In the presence of LBP and LPS, the onset of tumor necrosis factor-α (TNFα) production occurred earlier and at an LPS threshold dose that was as much as 1,000-fold lower for both types of LPS. In rabbit alveolar macrophages treated with LBP and LPS, TNFα mRNA appeared earlier, reached higher levels, and had a prolonged half-life as compared with LPS treatment alone. Neither LPS nor LPS and LBP affected pH_i or $[Ca_i^{++}]$ in alveolar macrophages. Specific monoclonal antibodies to CD14, a receptor that binds LPS/LBP complexes, inhibited TNFα production by human alveolar macrophages stimulated with LPS alone or with LPS/LBP complexes, indicating the importance of CD14 in mediating the effects of LPS on alveolar macrophages. Thus, immunoreactive LBP accumulates in lung lavage fluids in patients with lung injury and enhances LPS-stimulated TNFα gene expression in alveolar macrophages by a pathway that depends on the CD14 receptor. LBP may play an important role in augmenting TNFα expression by alveolar macrophages within the lungs. (*J. Clin. Invest.* 1992. 90:2209–2219.) Key words: alveolar macrophages • CD14 receptor • endotoxin • endotoxin binding protein • tumor necrosis factor

Introduction

Lipopolysaccharide (LPS) binding protein (LBP)[1] is a glycoprotein that is present in the plasma of normal animals and humans (1, 2). During acute-phase reactions, the plasma concentration of LBP rises by > 10-fold. In rabbits, LBP is produced in hepatocytes as a 50-kD protein that is glycosylated before secretion and circulates as a 60-kD glycoprotein (3). Previous studies have indicated that the LBP molecule contains a high-affinity binding site that binds the lipid A portion of LPS with 1:1 stoichiometry and K_d's in the nanomolar range (4). LBP opsonizes LPS-bearing particles, enhancing their association with the surface of monocyte-derived macrophages (5). Binding of LPS/LBP complexes to monocytes occurs via the CD14 antigen, which is present on monocytes, macrophages, and stimulated PMN (6).

Several lines of evidence suggest that LBP may play an important role in the host response to endotoxin. LBP lowers the threshold concentration at which LPS from smooth and rough forms of bacteria initiate the secretion of tumor necrosis factor-α (TNFα) by cells of the monocyte/macrophage lineage (7). Immunodepletion of LBP from normal rabbit plasma markedly attenuates LPS-induced TNFα production in rabbit whole blood (7). Antibodies to the CD14 receptor on monocytes block the TNFα production in human whole blood in response to LPS (6). Transfection of CD14 into the murine pre-B cell line 70Z/3 enhances IgM surface expression in response to LPS and LBP by several orders of magnitude (8). Thus, the LBP/CD14-dependent pathway appears to play an important role in mediating LPS-induced cytokine production by monocytic cells.

Our goal in the present study was to determine the potential relevance of this LBP/CD14 pathway for inflammatory reactions in the lungs. Normally, the lung airspace is isolated from the plasma compartment by a relatively impermeable epithelial barrier. During acute lung injury, such as that which occurs in patients with the adult respiratory distress syndrome (ARDS), the epithelial barrier is damaged and permeability increases markedly, allowing the movement of plasma proteins into the alveolar spaces (9). In these patients, production of cytokines such as TNFα in the lungs during lung injury may contribute to local and systemic manifestations of disease. Therefore, we investigated two questions: first, whether LBP is

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1. *Abbreviations used in this paper:* ARDS, adult respiratory distress syndrome; BCECF/AM, 2',7'-bis-(2-carboxyethyl)-5-(and-6)carboxyfluorescein acetoxymethyl ester; LBP, lipopolysaccharide binding protein; SPF, specific pathogen free; TNFα, tumor necrosis factor-α.

present in the alveolar spaces normally or when epithelial permeability is altered by acute lung injury; and second, whether LBP augments LPS-induced TNFα production by rabbit and human alveolar macrophages.

Methods

Reagents. Rabbit LBP was isolated from acute-phase rabbit serum as previously described (1). The final LBP preparation was sterile by culture on blood agar and gave a negative reaction for endotoxin contamination in a chromogenic limulus assay (Whittaker M.A. Bioproducts, Boston, MA). The LBP was aliquotted and stored frozen at −70°C before use. Endotoxin preparations (LPS) from *Salmonella minnesota Re595* (List Biologicals, Campbell, CA) and *Escherichia coli O111:B4* (Sigma Chemical Co., St. Louis, MO) were solubilized at 5 mg/ml in 10 mM EDTA by sonication, aliquotted, and stored frozen at −70°C. For each assay, an aliquot of stock LPS was thawed, sonicated for 10 s, and then diluted to the appropriate concentrations in 0.9% NaCl containing 10 mM Hepes buffer. Murine monoclonal antibodies 3c10 (anti-CD14) and 60.3 (anti-CD18) were the respective gifts of Wes Van Voorhis, Seattle, WA and Pat Beatty, Seattle WA. Murine monoclonal antibodies 28C5 (anti-CD14), 1E8 (anti-LBP), and 18G4 (anti-LBP) were generated by Didier LeTurcq from recombinant human soluble CD14 and recombinant human LBP.

Measurement of LBP in lung lavage fluids. Bronchoalveolar lavage fluid was obtained as described from two healthy volunteers who were free of lung disease (10) and from six patients with ARDS who were mechanically ventilated (11). ARDS was defined as the presence of critical hypoxemia on supplemental oxygen, diffuse pulmonary infiltrates, normal pulmonary capillary wedge pressure, and no other obvious cause for these findings. Lavage fluids from normal volunteers and patients with ARDS were stored frozen (−70°C) until analyzed.

Immunoreactive LBP was measured in bronchoalveolar lavage fluid from normal volunteers and patients with ARDS using two different methods: immunoprecipitation and enzyme immunoassay. LBP was immunoprecipitated using polyclonal anti-rabbit LBP antibody prepared by immunizing goats or rats with purified rabbit LBP as described (1). Aliquots (850 μl) of the six ARDS and the two normal lavage fluids were mixed with undiluted goat anti-rabbit LBP antiserum (150 μl) (15% vol/vol) and incubated overnight at 4°C. This antiserum concentration had previously been shown to give optimal immunoprecipitation results (1). To test the reproducibility of the results, the two ARDS lavage fluids with the highest protein concentrations (nos. 4 and 6) were also incubated with a rat anti-rabbit LBP antibody. After the overnight incubation, the fluids were spun at 12,000 *g* in a microcentrifuge at 4°C for 30 min to pellet the precipitates. The precipitates were washed once in buffer containing 150 mM NaCl, 50 mM Hepes, 0.2% Tween-20 (Bio-Rad Laboratories, Richmond, CA), then sonicated for 60 s, and respun at 12,000 *g* for 60 min. The resulting precipitates were washed again in H_2O and respun for an additional 60 min. The final precipitates were resuspended in Laemmli reducing buffer containing 5% β-mercaptoethanol and heated at 72°C for 30 min, then at 100°C for 2 min. The proteins were resolved by electrophoresis through 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes by electroblotting for 12 h at 4°C. The nitrocellulose membranes were incubated overnight in blocking buffer containing 1% gelatin, 3% bovine serum albumin, 0.05% Tween-20 and then reacted with purified goat anti-rabbit LBP (1:400 dilution) for 2 h at 21°C. The membranes were washed, incubated with recombinant ^{125}I-protein G (New England Nuclear, Boston, MA) for 30 min, washed thoroughly, and analyzed by autoradiography overnight at −70°C using XOMAT-AR film (Eastman Kodak Co., Rochester, NY).

LBP also was measured quantitatively in the same bronchoalveolar lavage fluids by enzyme immunoassay using murine monoclonal antibodies raised to purified human LBP. Microtiter plate wells were coated overnight with a murine monoclonal anti-LBP antibody (1E8), then washed three times and blocked with casein. Lavage fluids (200 μl) were added to each well and incubated for 1 h at 37°C. The wells were washed twice and incubated with a different biotinylated murine monoclonal anti-LBP antibody (18G4). The wells were washed again and the signal was developed by incubating with streptavidin-biotin-horseradish peroxidase complex for 30 min at 37°C, followed by *o*-phenylenediamine for 30 min and then 4.0 N H_2SO_4 to stop the reaction. The LBP concentration was determined by measuring the OD 490 nm in each well and comparing the results with a standard curve using purified human LPB. The assay is linear over the range of 30–250 ng LBP per well.

Cell recovery. Human alveolar macrophages were recovered by bronchoalveolar lavage from normal human volunteers who gave written informed consent as previously described (10). Briefly, the oropharynx was anesthetized with topical lidocaine and a flexible fiberoptic bronchoscope (model FB15A; Pentax Precision Instrument Co., Orangeburg, NJ) was passed into the lower airway. Single subsegments of both the right middle lobe and the lingula were lavaged with five separate 30-ml aliquots of sterile pyrogen-free 0.9% NaCl and each aliquot was recovered by gentle suction. The cells were recovered by centrifugation and the lavage fluids were cultured quantitatively on blood agar to assess the degree of bacterial contamination. The cells were washed twice in LPS-free RPMI-1640 media (Whittaker M.A. Bioproducts) containing 10 mM Hepes buffer, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin (complete RPMI-1640), counted, and suspended in complete RPMI-1640 media at 1×10^6 alveolar macrophages/ml before use in the assays.

Rabbit alveolar macrophages were recovered from specific pathogen-free (SPF) rabbits by bronchoalveolar lavage. Female rabbits weighing 1.5–2.0 kg were obtained from Western Oregon Rabbitry, Eugene, OR and monitored in the vivarium of the Seattle VA Medical Center for 1 wk before use. The rabbits were anesthetized deeply with intravenous pentobarbital and then exsanguinated by closed cardiac puncture. The thorax was opened and the lungs were lavaged in situ at a transpulmonary pressure of ~ 20 cm H_2O with five separate 50-ml aliquots of sterile, pyrogen-free 0.9% NaCl containing 0.6 mM EDTA. The lavage aliquots from each animal were pooled and the cells were pelleted by centrifugation at 200 *g* for 15 min. The cells were washed twice in complete RPMI-1640. Total cell counts were performed in a hemacytometer. Differential counts were performed on cytospin preparations stained with a modified Wright Giemsa stain (Diff-Quik, Scientific Products Co., McGaw Park, IN). Aliquots of the fresh lavage fluids (10 and 100 μl) were cultured quantitatively on blood agar to assess sterility. We used SPF rabbits because preliminary studies showed that the bronchoalveolar lavage fluids of non-SPF rabbits often were contaminated with low numbers of Gram-negative bacteria. The lavage fluids of the SPF animals were almost always sterile. Only alveolar macrophages from SPF animals with sterile lavage fluids were used in these studies.

Recovery of total cellular RNA and Northern blotting analysis. Total cellular RNA was recovered from rabbit alveolar macrophages by CsCl density gradient centrifugation using modifications of previously described methods (12, 13). Briefly, alveolar macrophages were lysed in a buffer containing 4.0 M guanidine thiocyanate, 25 mM trisodium citrate, 0.5% sodium laroyl sarcosine, 10 mM EDTA, 0.7% 2-mercaptoethanol, and 0.33% antifoam A (Sigma Chemical Co.). The lysates were underlayered with 5.7 M CsCl and centrifuged for 12 h at 110,000 *g* in an ultracentrifuge using either a Ti50 or a Ti75 rotor (Beckman Instruments Inc., Palo Alto, CA). The RNA pellets were solubilized in 10 mM Tris/0.1 mM EDTA and purified by phenol-cloroform extraction followed by precipitation in 70% ethanol/0.3 M sodium acetate. The RNA concentration was measured by spectrophotometry at 260 nM. The recovery of total RNA averaged ~ 3–5 μg of RNA/10^6 alveolar macrophages. The total cellular RNA (15 μg per lane) was denatured in 1 M glyoxal containing 50% DMSO and 10 mM sodium phosphate and electrophoresed through 1.2% agarose for 12 h at 1.0 V/cm. The quality of the RNA was assessed routinely by staining the gels in 0.01% acridine orange and examining the 28 S and 18 S RNA bands by ultraviolet fluorescence. The RNA was transferred to nylon mem-

branes (Biotrans, ICN Biomedicals, Inc., Irvine, CA) by overnight capillary transfer using 3 M NaCl containing 0.3 M trisodium acetate and then cross-linked to the membranes by ultraviolet irradiation. The membranes were hybridized at 60°C for 12 h using antisense oligonucleotide probes for rabbit TNFα and glyderaldehyde-3-phosphate dehydrogenase that were labeled with [α^{32}P]ATP (New England Nuclear) using Klenow's fragment of DNA polymerase exactly as described (12). The resulting hybridized membranes were developed by autoradiography at −70°C for up to 24 h using an intensification screen and Kodak X-OMAT AR film. The Northern analyses were quantified either directly using an automated phosphor-imaging system (PhosphorImager 400A Molecular Dynamics, Sunnyvale, CA) to count the radioactivity of each individual lane (14), or indirectly by performing videodensitometry of the images on the exposed xray film (Visage 2000, Bio Image Products, Millipore Corp., Ann Arbor, MI).

Localization of LBP mRNA in tissue specimens using polymerase chain reaction (PCR). To determine the cellular localization of mRNA for LBP, we used the polymerase chain reaction to amplify mRNA transcripts for LBP in total cellular RNA from rabbit liver, lung, and alveolar macrophages. A localized inflammatory reaction was produced in a normal SPF rabbit by injecting 1.0 ml of 3% $AgNO_3$ (wt/wt) in sterile pyrogen-free water subcutaneously between the shoulder blades as described (1). The animal was then killed 20 h later. The liver was removed aseptically and a portion was minced and immersed immediately into ice cold lysis buffer. The lungs were lavaged as described, and then slices of the left lower lobe were placed in ice cold lysis buffer. The lavage cell pellets were washed twice and then lysed in cold lysis buffer. The liver and lung slices were homogenized in cold lysis buffer, and then the total cellular RNA was recovered from the liver, lung and alveolar macrophage specimens as described above. The presence of LBP mRNA transcripts was analyzed with the PCR using the GeneAmp method (Perkin Elmer Cetus, Norwalk CT). The RNA was reverse transcribed using M-MLV reverse transcriptase, then amplified using AmpliTaq DNA polymerase in the presence of a sense/antisense primer pair specific for the coding region of the rabbit LBP cDNA sequence (predicted size 299 bases). For comparison, we used a sense/antisense primer pair specific for the coding sequence of the rabbit IL8 cDNA (predicted size 306 bases) because IL-8 mRNA is known to be produced in liver (15) and alveolar macrophages (16). The rabbit IL8 cDNA was isolated from a cDNA library that we had prepared from LPS-stimulated rabbit alveolar macrophages and had a coding sequence identical to that reported for rabbit IL-8 cloned from rabbit splenocytes (17). The cycling protocol consisted of melting at 94°C for 1 min, annealing at 55°C for 2 min, and extending at 72° for 3 min. After 30 amplification cycles, the reaction products were subjected to electrophoresis in 6% acrylamide and visualized by staining with ethidium bromide. The PCR reactions were performed simultaneously using RNA from the acute-phase rabbit liver, lungs, and alveolar macrophages. Additional tubes were included using RNA recovered from normal rabbit alveolar macrophages incubated for 4 h in the presence or absence of 1 ng/ml Re595 LPS. This concentration of LPS was chosen because it was shown to produce marked accumulation of cytokine mRNA at 4 h (TNFα).

The identities of the LBP and IL8 PCR products were verified in three ways. First, the PCR products gave bands of the predicted size on polyacrylamide gel electrophoresis. Second, restriction digestion of the PCR products using restriction enzymes with sites within the predicted target sequences gave digestion products of exactly the predicted size. Third, the PCR products were cloned into the plasmid vector PCR1000 (In Vitrogen Co, San Diego, CA), which was then inserted into *E. coli* by electroporation. The plasmid was then harvested and the insert was sequenced using the Sequenase reaction system (U.S. Biochemical Corp., Cleveland, OH). The observed sequences of the rabbit LBP and IL-8 inserts matched the known base sequences of the predicted target sequences.

When the PCR primers were used to amplify the rabbit liver RNA preparations without first conducting the reverse-transcription step, no signal for LBP was detected, making it unlikely that trace amounts of genomic DNA that might have been present in the samples could have been the target of the amplification reaction.

Measurement of TNFα. TNFα was measured in a cytotoxicity assay using the L929 cell line as described (12, 18). L929 cells were suspended in RPMI media containing 10 mM Hepes, 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and actinomycin D (1.0 μg/ml) and aliquotted in 96-well microtiter plates at 2×10^4 per well. The cells were allowed to adhere to the plates for 2 h at 37°C in 5% CO_2. Then aliquots of each sample (5 μl) were added to duplicate wells and titered by serial threefold dilutions. After incubation for 18 h at 37°C/5% CO_2, the residual cells were fixed and stained with 0.2% crystal violet/3.7% formalin, and the absorbance in each well was measured spectrophotometrically (model MR5000, Dynatech Labs, Inc., Chantilly, VA). The cytolytic activity in each sample was quantitated by comparing the half-maximal lytic concentration in the samples with that in wells containing serial threefold dilutions of a standard of known TNFα activity prepared from LPS-treated RAW 264.7 cells (5×10^4 U/ml). The TNFα activity in the RAW cell standard was calibrated using a human recombinant TNFα standard obtained from the National Institute for Biological Standards and Control, Hertfordshire, UK.

Measurement of CD14 expression on alveolar macrophages and blood monocytes. We measured the expression of the CD14 receptor on the surface of human alveolar macrophages (1×10^6 cells/ml in complete RPMI-1640 media) stimulated with LPS with or without added LBP. For comparison, peripheral blood mononuclear cells were recovered on the same day by density gradient centrifugation (Mono-poly Resolving Medium, Flow Laboratories, Inc., Costa Mesa, CA) from the same volunteers who underwent bronchoalveolar lavage and treated in the same manner as the alveolar macrophages. The alveolar macrophages and blood mononuclear cells were incubated in 1.5 ml polypropylene tubes (to minimize adherence) with O111:B4 LPS (10 ng/ml) with or without LBP (100 ng/ml) at 37°C in 5% CO_2/air for 4 h. CD14 was measured by indirect immunofluorescence using the murine antibody My4 (Coulter Laboratories, Hialeah, FL) as the primary antibody and goat anti-mouse IgG as the detecting antibody (phycoerythrin conjugate, Accurate Chemical & Scientific Corp., Westbury, NY). For comparison, the cells were labeled with murine 60.3, which labels CD18 (19). The alveolar macrophages and blood mononuclear cells were washed twice, suspended at 5×10^6/ml in HBSS containing 0.1% bovine serum albumin and 0.1% NaN_3, and incubated on ice for 30 min with the primary antibody at a dilution of 1:100, followed by a 30-min incubation with the secondary antibody at a dilution of 1:500. After the labeling procedure, the cells were washed and then analyzed using a FACS Scan instrument (Becton Dickinson & Co., Mountain View, CA) with the gatings set to identify monocytes and macrophages.

Measurement of intracellular pH and calcium. Intracellular pH in rabbit alveolar macrophages was measured using the fluorescent intracellular probe, 2',7'-bis-(2-carboxyethyl)-5-(and-6)carboxyfluorescein (BCECF; Molecular Probes, Inc., Eugene, OR) (20). Alveolar macrophages were suspended at 1.5×10^6/ml in complete RPMI 1640 and loaded with 0.5 μM BCECF/acetoxymethyl (AM) ester for 1 h at 37°C, washed twice, and resuspended in buffer containing 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, 20 mM Hepes, and then placed in a quartz cuvette in a model LS5B fluorescence spectrometer (Perkin Elmer Corp., Norwalk, CT) equipped with a thermostatically controlled water-jacketed cell holder (37°C). The excitation wavelength was set at 505 nm and emission was scanned between 510 and 600 nm at 3-min intervals before and after the addition of LPS or LBP. The height of the emission peak at 530 nm was recorded at each time. The height of the 530 nm peak decreases as pH falls. The system was calibrated over the pH range 6.5–8.0 by incubating alveolar macrophages in calibrating buffers of known pH containing 25 mM NaCl, 120 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, 20 mM Hepes, and nigericin 10 μg/ml (Sigma Chemical Co.) to allow equilibration of intracellular and extracellular pH. Using rabbit alveolar macrophages, the plot of pH vs. BCECF emission intensity at 530 nm was linear over the pH range 6.5–7.5. A change in

Table I. Bronchoalveolar Lavage Findings in the Patient Population

Patient	Risk factor	Total protein	Total WBC	Alveolar macrophages	Neutrophils	Lymphocytes	Eosinophils	Outcome
		mg/dl	*×10⁴/ml*	%	%	%	%	
1	OD	33.1	93.6	63.5	34.5	2.0	0.0	D
2	OD	7.6	80.6	73.0	25.5	1.5	0.0	L
3	OD	87.5	73.0	78.0	17.5	2.0	2.5	L
4	trauma	244.9	168.6	5.0	94.0	0.5	0.5	D
5	sepsis	31.2	52.6	5.0	95.0	0.0	0.0	D
6	trauma	53.8	22.8	16.5	78.0	1.5	4.0	L
NL	—	10.4	10.0	96.0	1.0	3.0	0	
NL	—	9.2	7.5	94.0	1.0	5.0	0	

Abbreviations: D, died; L, lived; NL, normal volunteer; OD, drug overdose.

emission intensity of 10 U corresponds to a change in intracellular pH of 0.1 U. This assay is sensitive to changes in intracellular pH of ~ 0.01 unit around pH 7.1. These findings agree closely with a prior report using rabbit alveolar macrophages (21).

To measure changes in intracellular calcium, alveolar macrophages were suspended at 1.5×10^6/ml in Hank's balanced salt solution (HBSS) containing Ca^{++} and Mg^{++}, incubated with 1.0 μM Fura-2/AM (Molecular Probes, Inc.) for 1 h at 37°C, washed twice, and resuspended in HBSS at 1.0×10^6/ml. As for pH measurements, the cells were placed in the fluorescence spectrophotometer adjusted to a constant temperature of 37°C. Excitation scans were performed every 3 min over the wavelength range 320–440 nm, and emission was monitored at 510 nm. The ratio of the heights of the 510-nm emission peaks at the excitation wavelengths of 347 and 389 nm was recorded and the intracellular calcium concentration was calculated as described (22). Values for R_{max} and R_{min} were determined from additional macrophages incubated with ionomycin (5 μg/ml) to increase intracellular calcium, or ionomycin with EGTA to minimize intracellular calcium, as described (22). This method is sensitive to changes of ~ 20 nM in intracellular free calcium concentration.

Results

Detection of LBP in lung fluids. To determine whether LBP is present in the airspaces of normal or inflammed lungs, we studied bronchoalveolar lavage fluids from two normal volunteers and six subjects with acute lung injury, ARDS. The characteristics of the lavage fluids are shown in Table I. The normal fluids contained > 90% viable alveolar macrophages and had protein concentrations of 10.4 and 9.2 mg/dl. In contrast, the lavage fluids of the patients with ARDS contained an abundance of neutrophils (range 17.5–95%) and five of the six contained an increased total protein concentration (range 7.6–244.9 mg/dl). These findings are consistent with prior observations in patients with severe lung injury (9, 11).

Equal volumes of the lavage fluids (850 μl) were subjected to immunoprecipitation with goat anti-rabbit LBP polyspecific antisera and the precipitates were analyzed by SDS-PAGE and Western blotting using polyspecific goat anti-LBP antiserum. For comparison we also immunoprecipitated the two fluids with the highest proteins using a different polyspecific anti-rabbit LBP antiserum raised in rats. The results are shown in Fig. 1. A small amount of immunoreactive LBP was detectable in the lavage fluids of the two normal volunteers (lanes *7* and *8*) and in the ARDS fluids with low protein concentrations (patients 2 and 5, lanes *2* and *5*). In contrast, greater amounts of immunoreactive LBP were present in the lavage fluids of the patients with ARDS with the higher protein concentrations (patients 1, 3, 4, and 6 shown in the corresponding lanes). Immunoprecipitation of the lavage fluids from patients 4 and 6 with the goat and the rat anti-LBP antibodies gave similar immunoprecipitation results (Fig. 1, lanes *4* and *6* vs. *9* and *10*). Immunoreactive bands of lower molecular weight were not detected in the lavage fluids, suggesting that the immunoreactive LBP detected by this method had not been degraded in the lungs by proteolytic cleavage.

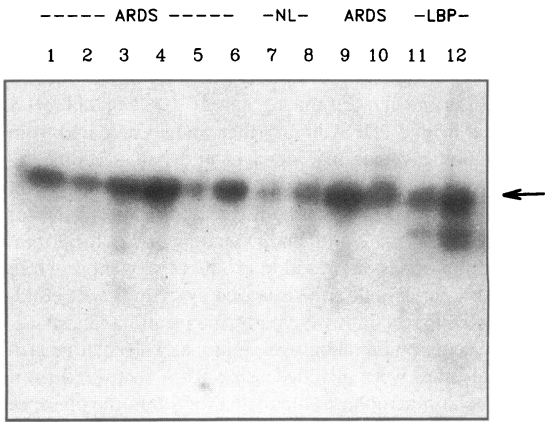

Figure 1. Immunoprecipitation of LBP from bronchoalveolar lavage fluids from six patients with ARDS (lanes *1–6*) and two normal volunteers (lanes *7* and *8*). Lanes *1–6* correspond to the numbering of the patients in Table I. The lavage fluids were incubated at 4° overnight with 15% (vol/vol) goat or rat anti-rabbit LBP antibody. The precipitates were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions, electroblotted to a nitrocellulose membrane, and incubated with a different goat anti-rabbit LBP antibody, followed by ^{131}I-protein G. Lanes *1–8* show the results of lavage fluids precipitated with goat anti-rabbit LBP antibody. Lane *11* shows 100 ng/ml purified rabbit LBP immuno-precipitated with this same antibody. Lanes *9* and *10* show the results of precipitating ARDS fluids from the two patients shown in lanes *4* and *6* (ARDS patients 4 and 6) with a rat anti-rabbit LBP antibody. Lane *12* shows the results of precipitating 100 ng/ml purified rabbit LBP with this antibody. All of the immunoprecipitations were performed at the same time under the same conditions. The arrow indicates 60 kD.

Measurement of immunoreactive LBP by immunoassay using these same lavage fluids gave results that supported the immunoprecipitation experiments, although the immunoassay was less sensitive than the immunoprecipitation. The levels of LBP were at or below the detection limit of this assay (~ 40 ng/ml) in the two normal fluids (Fig. 1, lanes *7* and *8*) and in the fluids that gave the lightest bands by immunoprecipitation (Fig. 1, lanes *1, 2,* and *5*). The fluids from subjects 3, 4, and 6 contained 150, 600, and 120 ng/ml LBP, respectively.

Tissue localization of LBP mRNA. To determine whether LBP might be produced in lung cells, we used the PCR method to identify mRNA transcripts coding for LBP in total cellular RNA from rabbit lung homogenate, rabbit alveolar macrophages, and rabbit liver, where LBP is known to be produced (3) (Fig. 2). Liver mincings, lung mincings, and alveolar macrophages were recovered from a rabbit 24 h after the subcutaneous injection of 1.0 ml of 3% $AgNO_3$ to induce an acute phase response. For comparison, we studied RNA derived from normal rabbit alveolar macrophages (Fig. 2, lanes *7* and *8*) and rabbit alveolar macrophages that were incubated for 4 h with 1 ng/ml Re595 LPS (Fig. 2, lanes *9* and *10*). This concentration of LPS is sufficient to cause marked accumulation of cytokine mRNA by 4 h. As seen in Fig. 2, a product with the expected size of the LBP target sequence (299 bp) amplified in the rabbit liver RNA, but not in the RNA from the rabbit lung mincings or the alveolar macrophages. Direct sequencing of this PCR product proved that it had the expected LBP sequence. The LBP product also was not detected in the RNA of rabbit alveolar macrophages that had been incubated for 4 h with LPS. By contrast, the IL-8 product of the expected size (306 bp) was detected in liver, lung, and alveolar macrophages.

Effect of LPS and LPS/LBP on TNFα production by human and rabbit alveolar macrophages. As LBP accumulates in lung fluids during acute lung injury, we next examined the dose and time dependency for LPS-induced TNFα release from human and rabbit alveolar macrophages in the presence and absence of purified rabbit LBP. R-form (Re595) and S-form (O111:B4) LPS were added to rabbit alveolar macrophages in concentrations ranging from 10 pg to 1 μg/ml in the absence or presence of a constant ratio of purified rabbit LBP (12:1 LBP/LPS, wt/wt). The TNFα present in the cell free supernatants was measured at 4, 8, or 16 h after the addition of LPS and LBP. The data shown in Fig. 3 demonstrate that the addition of LBP lowered the threshold dose of both forms of LPS by up to 1,000-fold and also accelerated the rate of TNFα production. This effect was detectable 4 h after stimulation of the macrophages and was still present at 16 h. In the absence of LPS, LBP did not cause the release of any detectable TNFα from the alveolar macrophages. Heating the LBP to 90°C for 15 min destroyed the ability of the preparation to enhance the effect of LPS. Neither bovine serum albumin (10 μg/ml) nor rabbit IgG (10 μg/ml) affected the LPS dose response for TNFα production by rabbit alveolar macrophages.

The addition of purified rabbit LBP to human alveolar macrophages together with Re595 LPS produced effects similar to those noted for rabbit alveolar macrophages (Fig. 4). In the absence of LBP, the threshold for TNFα production was ~ 1.0 ng/ml for Re595 LPS. In the presence of LBP, the threshold for TNFα production in response to Re595 LPS was lowered to between 10 and 100 pg/ml for Re595 LPS and the rate of TNFα production was increased. The maximal TNFα concentrations produced by human alveolar macrophages treated

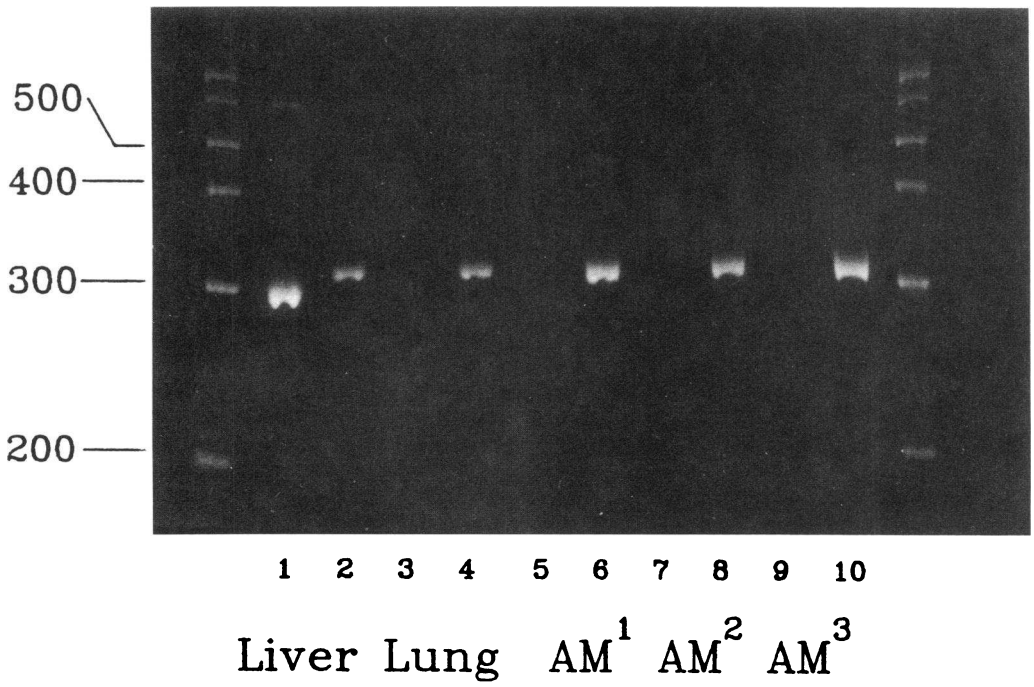

Figure 2. Analysis of LBP and IL-8 mRNA transcripts using PCR. Total cellular RNA from liver tissue (lanes *1* and *2*), lung tissue (lanes *3* and *4*) and alveolar macrophages (AM^1, lanes *5* and *6*) was recovered from a rabbit 24 h after the subcutaneous injection of 1.0 ml of 3% $AgNO_3$ to induce an acute phase response. For comparison total cellular RNA samples from normal rabbit alveolar macrophages (AM^2, lanes *7* and *8*) and alveolar macrophages incubated for 4 h with *S. minnesota* Re595 LPS 1.0 ng/ml (AM^3, lanes *9* and *10*) also were studied. The PCR reactions were performed using primer sets specific for the coding region of the cDNAs for rabbit LBP and rabbit IL-8. After 30 cycles of PCR, the products were analyzed by electrophoresis in a 6% polyacrylamide gel and stained with ethidium bromide. Lanes *1, 3, 5, 7,* and *9,* LBP primer product. Lanes *2, 4, 6, 8,* and *10,* IL-8 primer product. The LBP product (expected size 299 bp) was visible only in the liver RNA sample (lane *1*). The IL-8 product (expected size 306 bp) is visible in each of the samples. In the absence of RNA no signal was detected with either set of primers. In the absence of the reverse transcriptase step, no signal was detected for LBP in the liver RNA specimen. The base ladder standard is shown in the end lanes for comparison.

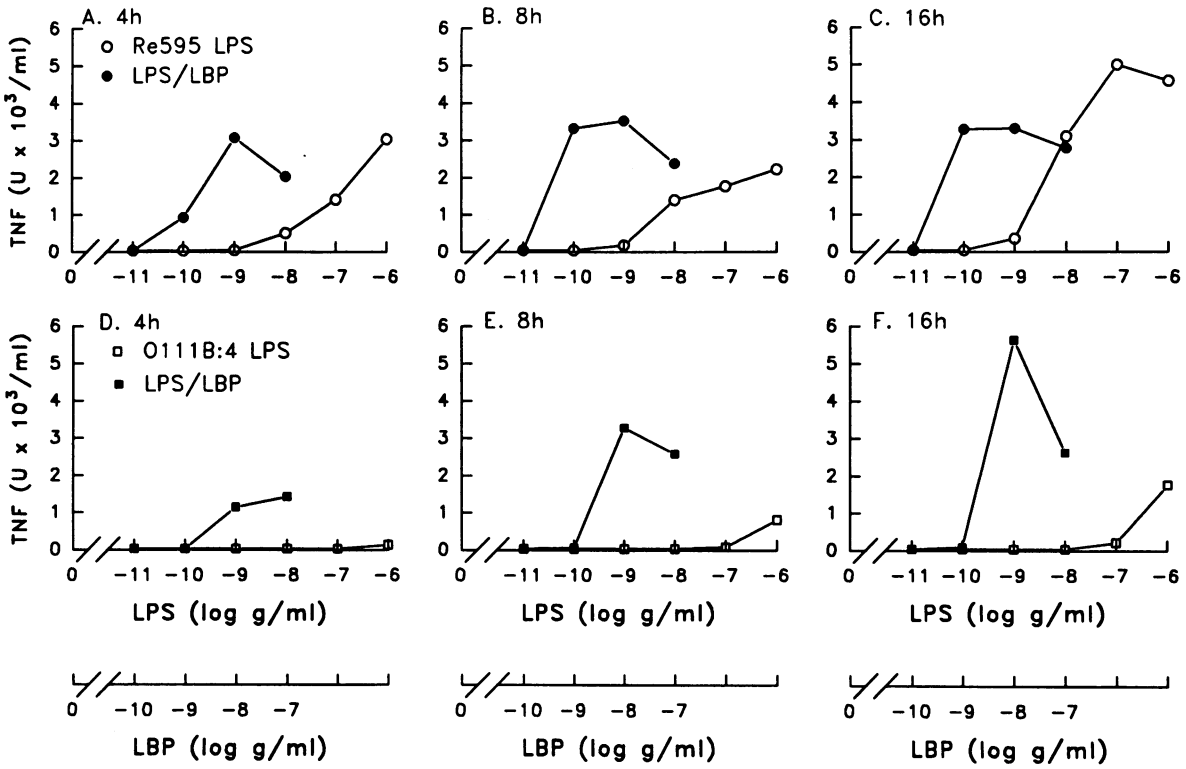

Figure 3. The production of TNFα by rabbit alveolar macrophages stimulated with LPS and purified rabbit LBP. Rabbit alveolar macrophages were incubated for 4 h (*A* and *D*), 8 h (*B* and *E*), or 16 h (*C* and *F*) with increasing concentrations of LPS in the absence or presence of a 12-fold higher concentration (wt/wt) of purified rabbit LBP as indicated in the legend. LBP was not added at the highest concentrations of added LPS. (*A–C*) *S. minnesota* Re595 LPS (rough form) is shown. (*D–F*) *E. coli* O111:B4 LPS (smooth form) is shown. The addition of LBP with LPS caused TNFα production to begin sooner and at a lower LPS concentration than with LPS alone. LBP did not stimulate TNFα production in the absence of LPS. Data points are the means of duplicate TNFα measurements. This experiment was repeated four times with similar results using alveolar macrophages from different animals.

with LPS and LBP approached those seen with human alveolar macrophages incubated with a 100-fold higher concentration of the Re595 LPS.

We next sought to define the LBP dose response for enhancing LPS-induced TNFα release by alveolar macrophages. Concentrations of Re595 LPS (0.1 ng/ml) or O111B:4 LPS (1.0 ng/ml) that induced little or no TNFα release from rabbit alveolar macrophages during 18 h of incubation were mixed with varying amounts of LBP (0.012–120 ng/ml). TNFα release in the macrophage supernatants was measured 4 or 18 h

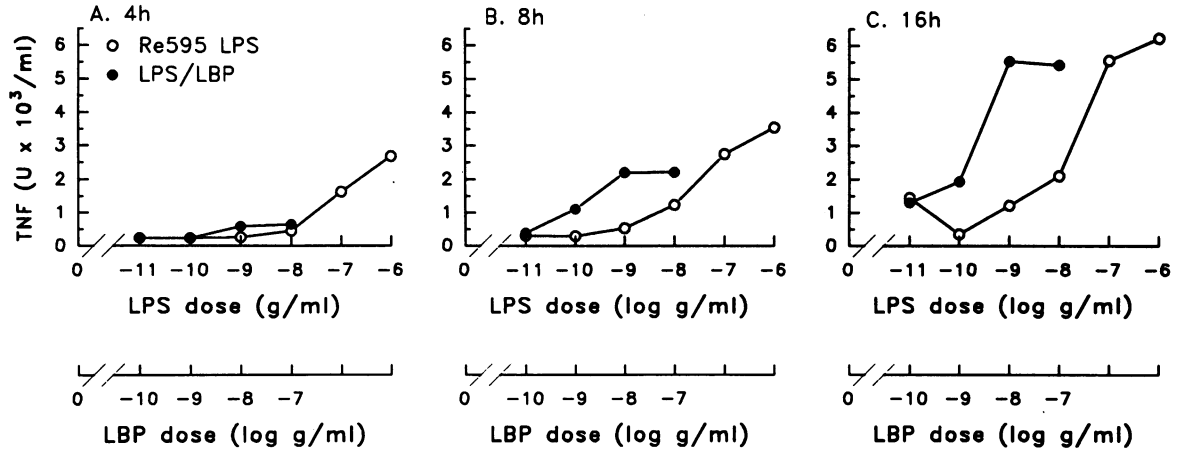

Figure 4. The production of TNFα by human alveolar macrophages stimulated with LPS and purified rabbit LBP. The format is the same as that in Fig. 3. Human alveolar macrophages were incubated with rough form *S. minnesota* Re595 LPS for 4, 8, or 16 h in the absence or presence of a 12-fold excess of LBP as indicated. As with rabbit alveolar macrophages, LBP caused TNFα production to begin sooner and at lower concentrations of LPS than with LPS alone. Data points are the means of duplicate TNFα measurements. This experiment was performed four times using alveolar macrophages from four different normal human volunteers with similar results.

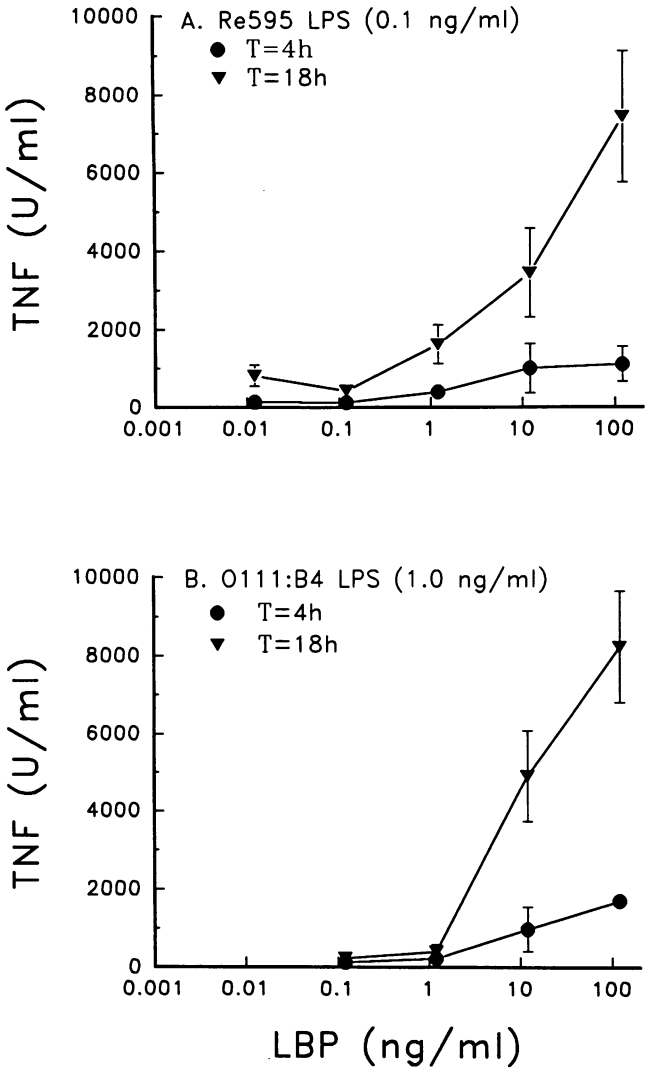

Figure 5. Dose response for rabbit alveolar macrophages to purified rabbit LBP in the presence of a subthreshold concentration of Re595 LPS. In this experiment rabbit alveolar macrophages were incubated with a constant dose of LPS that by itself did not induce TNFα production. LBP was added in increasing concentrations as shown on the horizontal axis to duplicate wells and TNFα determinations were performed in duplicate in each supernatant sample. (*A*) *S. minnesota* Re595 LPS (0.1 ng/ml). (*B*) *E. coli* O111:B4 LPS (1.0 ng/ml). LBP stimulated TNFα release in a dose- and time-dependent fashion. In the absence of LPS, the highest concentration of LBP tested (120 ng/ml) caused no release of TNFα from the alveolar macrophages. Data points are the means±SE of duplicate TNFα measurements in three different experiments using alveolar macrophages from three different SPF rabbits.

after the addition of LPS and LBP (Fig. 5). The addition of LBP caused a dose-dependent increase in TNFα production in response to each form of LPS. For Re595 LPS, TNFα production occurred at LBP/LPS ratios of 10:1 and greater. For O111:B4 LPS, TNFα production also began at a 1:10 ratio of LBP/LPS. In the absence of LPS, the maximal concentration of LBP (120 ng/ml) caused no detectable TNFα production during the 18-h incubation period.

Effect of LPS and LPS/LBP on TNFα mRNA in alveolar macrophages. In order to begin to define the mechanism by which LBP enhances LPS-induced TNFα production in rabbit alveolar macrophages, we measured the kinetics of TNFα mRNA in rabbit alveolar macrophages stimulated with Re 595 LPS with or without added LBP. The results of Northern blot analysis of total cellular RNA probed for TNFα mRNA are shown in Fig. 6. Treatment with 1 ng/ml Re595 LPS caused a progressive increase in the level of the TNFα mRNA signal over 8 h. In contrast, the addition of LBP with LPS caused the TNFα mRNA to appear earlier, to reach a higher steady-state level, and to decline faster than with LPS alone. LBP by itself did not stimulate alveolar macrophages to produce TNFα mRNA. In additional experiments, we added actinomycin D (5 μg/ml) to rabbit alveolar macrophages 4 h after the addition of either LPS (1 ng/ml) or LPS (1 ng/ml) plus LBP (10 ng/ml), then harvested total cellular RNA from parallel flasks of alveolar macrophages at sequential times for the measurement of the rate of decline of the TNF mRNA signal. With LPS alone, there was a steady decay in the TNFα mRNA levels, corresponding to a calculated half-life of 0.5 h by scanning densitometry and also by quantitative phosphorimaging (15). By contrast, after stimulation with LPS and LBP, the TNFα mRNA signal persisted for a longer time, corresponding to a calculated half-life of > 2.5 h.

Importance of CD14 for LPS-induced TNFα production by human alveolar macrophages. Because the CD14 receptor appears to be an essential component of the pathway by which LPS/LBP complexes stimulate blood monocytes, we sought to determine whether incubation of alveolar macrophages with either LPS alone or LPS with LBP regulates the expression of CD14 on alveolar macrophages and blood monocytes from the same individuals, and the effects of blocking CD14 with specific monoclonal antibodies on TNFα production. As seen in Fig. 7, alveolar macrophages express less CD14 than blood monocytes from the same individual. Incubating alveolar macrophages with LPS (10 ng/ml) or LPS (10 ng/ml) and LBP (100 ng/ml) for 4 h did not affect the expression of CD14. This dose of LPS exceeded by 10-fold the dose required to initiate transcription of the TNFα gene in rabbit alveolar macrophages (Fig. 6). In similar experiments, we also found no change in

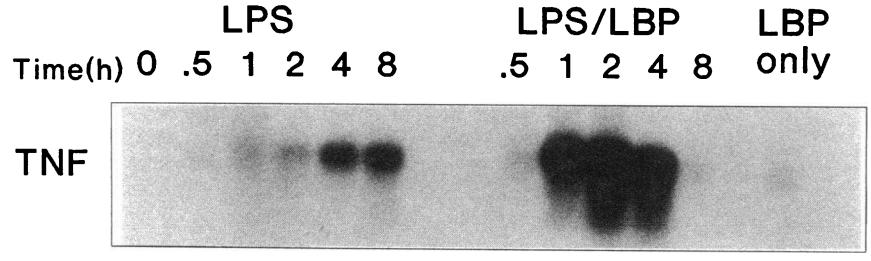

Figure 6. Effect of LPS and LBP on the induction of TNFα message in rabbit AM. AM were incubated for up to 8 h with either 1 ng/ml Re595 LPS alone (*left*), 1 ng/ml Re595 LPS and 12 ng/ml rabbit LBP (*middle*), or with 12 ng/ml LBP alone for 4 h (*right*). Total cellular RNA was recovered at the indicated times and Northern analysis was performed as described in Methods. The membranes were hybridized with a ^{32}P-labeled cDNA specific for rabbit TNFα and developed by autoradiography.

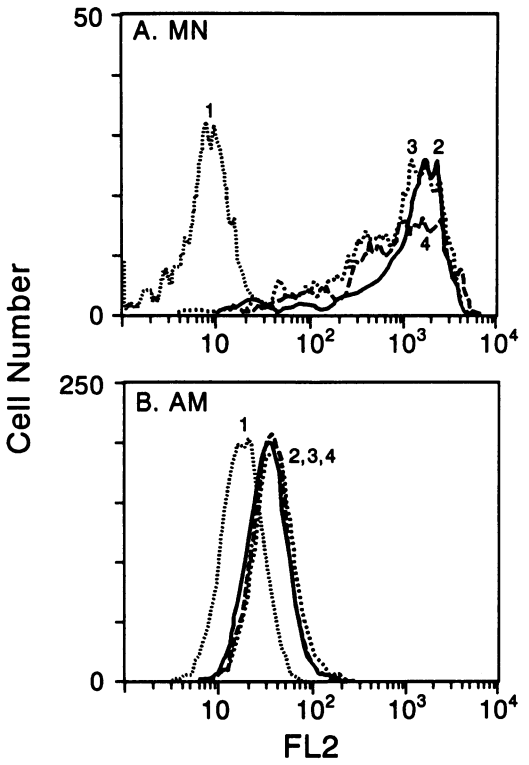

Figure 7. The effect of LPS and LPS/LBP on CD14 expression by human monocytes (*A*) and alveolar macrophages (*B*). The cells were labeled with MY4 by indirect immunofluorescence and analyzed by flow cytometry. The vertical axis shows cell number in each fluorescence channel; the horizontal axis is a $\log_{10}$ scale of red fluorescence intensity. In each panel the labels refer to cells treated as follows: *1*, untreated cells, 4 h, labeled with the second antibody only (goat anti-mouse IgG); *2*, untreated cells, 4 h; *3*, LPS 10 ng/ml, 4 h; *4*, LPS 10 ng/ml + LBP 100 ng/ml, 4 h.

the expression of CD14 on rabbit alveolar macrophages (not shown). In comparison, the CD14 fluorescence profile of blood monocytes from the same individual shifted slightly to the left after incubation with LPS and LPS/LBP, suggesting down-regulation of the CD14 receptor on some of the cells.

To test the importance of CD14 on mediating the responses of human alveolar macrophages to LPS and LPS/LBP complexes, we incubated alveolar macrophages with LPS or LPS/LBP in the presence of saturating concentrations of 28C5 (5 μg/ml), a specific murine monoclonal antibody raised against purified recombinant human CD14 that was expressed in CHO cells. This antibody labels human alveolar macrophages and blood monocytes, immunoprecipitates a single band consistent with CD14 from HL60 cells differentiated with $1,25(OH)_2$-vitamin D, and competes with radiolabeled 3C10, another anti-CD14 antibody (6), in labeling HL60 cells. As shown in Fig. 8, the antibody 28C5 inhibited TNFα production by human alveolar macrophages incubated with LPS alone, and completely blocked the enhancement of TNFα production caused by LBP. We obtained similar results in this system when we used the 3C10 antibody. The 28C5 antibody had no effect on the production of TNFα by alveolar macrophages in response to heat-killed *Staphylococcus aureus* (Fig. 8).

Effect of LPS and LBP on intracellular calcium and pH. We next asked whether the addition of LPS or LPS/LBP complexes might cause changes in intracellular Ca^{++} or pH in alveolar macrophages, as these events might contribute to signal transduction pathways that mediate cytokine production after ligation of CD14. As seen in Fig. 9, neither LPS nor LPS/LBP complexes affected either $[Ca_i^{++}]$ or pH_i in rabbit alveolar macrophages within the first 30 min of incubation. These experiments were conducted at the concentrations of LPS (1–10 ng/ml) and LBP (10–100 ng/ml) that were shown by Northern

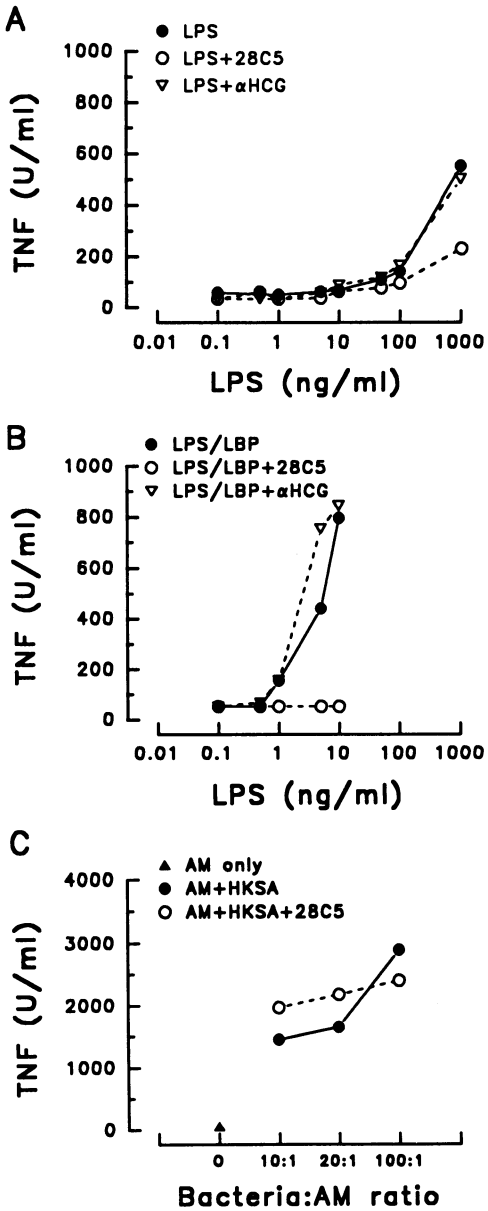

Figure 8. The effect of anti-CD14 antibody on the response of human alveolar macrophages to LPS and LPS/LBP. (*A*) Human alveolar macrophages were incubated for 8 h with increasing concentrations of LPS with or without the monoclonal antibodies 28C5 (5 μg/ml), which labels CD14, or an irrelevant antibody (anti-human chorionic gonadotropin) of the same isotype. (*B*) Alveolar macrophages from the same volunteer incubated with LPS and 10-fold higher concentrations of LPB (wt/wt) with or without 28C5 or anti-human chorionic gonadotropin. (*C*) Alveolar macrophages from the same volunteer incubated for 18 h with increasing concentrations of heat-killed *S. aureus* with or without 28C5. The inhibitory effects of 28C5 were tested in three experiments with alveolar macrophages from different volunteers with similar results.

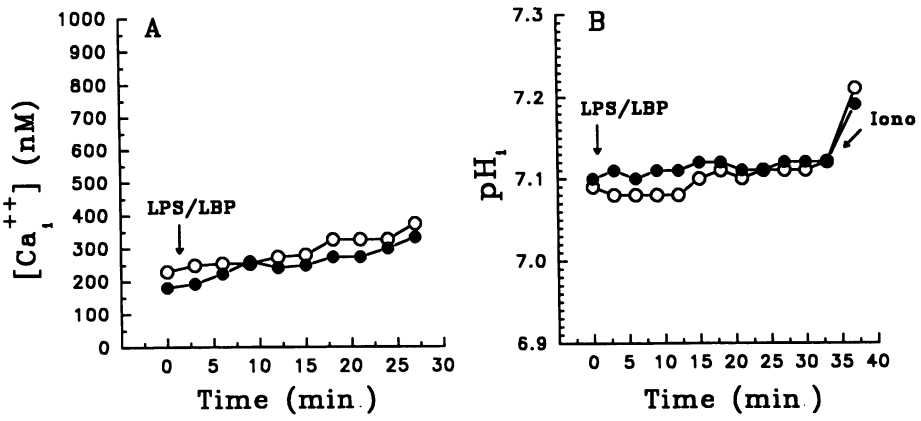

Figure 9. The effect of LPS and LBP on intracellular free calcium (*A*) and pH (*B*) in rabbit AM. (*A*) To measure intracellular free calcium, alveolar macrophages were loaded with 1.0 μM Fura2/AM then stimulated with Re595 LPS (1 ng/ml) and purified rabbit LBP (10 ng/ml). The excitation wavelength was set at 510 nm. The ratio of the emission intensity at 347/389 nm was used to calculate the intracellular calcium concentration, shown on the vertical axis. The open circles show the response of cells incubated without LPS or LBP. The response in cells treated with LPS alone (1 ng/ml) was identical to the data shown for untreated cells. When ionomycin was added (10 μg/ml), the intracellular calcium concentration increased abruptly to over 1 μM. (*B*) To measure intracellular pH, AM were loaded with 0.5 μm BCECF/AM and stimulated with Re595 LPS (1 ng/ml) and purified rabbit LBP (10 ng/ml). The excitation wavelength was set at 505 nm and the intensity of the emission peak at 530 was recorded. Intracellular pH, (*vertical axis*) was calculated from a standard curve derived by incubating additional cells in high potassium buffers of known pH. *Iono* denotes the addition of ionomycin (10 μg/ml) as a positive stimulus. The open circles show the response of cells in the absence of LPS and LBP. The response in cells treated with LPS alone (1 ng/ml) was identical to the data shown for untreated cells. The experiments in *A* and *B* were performed three different times using alveolar macrophages from different rabbits with similar results.

analysis to result in substantial TNFα message production by Re595 LPS alone and enhanced TNFα message production with added LBP (Fig. 3).

Discussion

The major goals of this study were first, to investigate whether immunoreactive LBP accumulates in fluid recovered from normal or injured human lungs, and second to investigate the effect of LBP on LPS-induced TNFα production by rabbit and human alveolar macrophages. The results indicate that immunoreactive LBP is normally present in very low concentrations in the alveolar fluid, and that its concentration rises substantially in patients with lung injury. In rabbits, LBP in lung fluids appears to originate from an extrapulmonary source, as we did not detect LBP mRNA in RNA extracted either from rabbit lung tissue or from normal or LPS-stimulated rabbit alveolar macrophages. LBP enhances the response of human and rabbit alveolar macrophages to LPS. In the presence of LBP and LPS, the maximal TNFα response by alveolar macrophages occurs earlier than with LPS alone and it also occurs at lower concentrations of both smooth and rough form LPS. The CD14 receptor plays an important role in mediating the effects of LPS and LBP on alveolar macrophages, as the effects of LPS and LBP can be inhibited by anti-CD14 monoclonal antibodies. Thus, LBP that accumulates in the lungs during lung injury may have an important regulatory effect on the response of alveolar macrophages to endotoxin that enters the airspaces.

The origin of the LBP in the lungs and the mechanism by which it enters the lungs is of considerable interest. The results of the experiments using the sensitive PCR method indicate that in rabbits the LBP present in lung lavage fluid probably derives from the liver and not from the lungs. Although alveolar macrophages are capable of producing a variety of proteins, mRNA transcripts for LBP were not detected in normal rabbit alveolar macrophages, alveolar macrophages obtained during a systemic acute-phase response, or alveolar macrophages stimulated with LPS. In addition, we have not been able to detect LBP activity by immunoassay in the supernatants of LPS-stimulated human or rabbit alveolar macrophages (not shown). The data confirms an earlier study in which LPB message was detected in rabbit liver RNA by Northern analysis (3). LBP (60 kD) is similar in molecular mass to albumin (67 kD), which constitutes ~ 50% of the protein in bronchoalveolar lavage fluid (23). When the permeability of the epithelial barrier in the lungs is altered, much higher molecular mass proteins, e.g., IgM (900 kD), that normally are excluded from the alveolar spaces are readily detectable in lavage fluid (9, 23, 24). Under these conditions, proteins of the size of LBP would be expected to pass rapidly from the plasma compartment into the alveolar spaces. These lines of evidence support the interpretation that LBP is synthesized in the liver and that it accumulates in the lungs as lung epithelial permeability increases in response to injury.

Our findings have important implications for the mechanisms that regulate the production of cytokines such as TNFα within the lungs during inflammatory reactions. TNFα is thought to be an important mediator of the sepsis syndrome in animal models (25–27), and sepsis syndrome both precedes and complicates ARDS in many patients (28). The alveolar macrophage plays a prominent role in the response of the lung to endotoxins because it responds to LPS by producing an array of cytokines that modify the inflammatory response. In addition to TNFα, these include IL-6 (29), IL-8 (30) and to a lesser extent IL-1β (31, 32). Although other cells of the alveolar environment, including epithelial cells (33), fibroblasts (34), endothelial cells (16), and mesothelial cells (35) have the capacity to produce some cytokines, particularly IL-8, the alveolar macrophage appears to be the primary cell in the airspace that recognizes and responds to LPS (reviewed in reference 35).

Our results suggest that LBP may play a major role in regulating the sensitivity of alveolar macrophages to LPS by both lowering the threshold and accelerating the time course of cytokine production. A previous study showed that LBP augments the response of elicited rabbit peritoneal exudate macrophages to smooth and rough form LPS (7). The present studies show

that LBP enhances the effect of LPS on a nonelicited resident macrophage population in the lungs of both rabbits, from which the LBP was derived, and normal humans. As lung macrophages may be an important local source of TNFα during acute lung injury, the accumulation of LBP in the inflammed airspaces may provide an endogenous mechanism for amplifying or regulating the response of lung macrophages to local concentrations of LPS within the lungs.

The intracellular pathways by which LBP accentuates the effect of LPS on alveolar macrophage warrant further study. The binding of LPS/LBP complexes to surface CD14 on monocytes appears to be the first step in the recognition of LPS/LBP complexes by monocytic cells (6). The present data confirm prior reports that CD14 is present on alveolar macrophages (36, 37), although the expression of CD14 on alveolar macrophages is considerably less than on peripheral blood monocytes. We found that LPS and LPS/LBP induce TNFα gene expression without affecting the density of CD14 on alveolar macrophages. We also found that specific monoclonal antibodies directed at epitopes on CD14 blocked the effect of LPS and LPS/LBP on inducing TNFα expression. Although other cell surface proteins have been identified that may contribute to the effects of endotoxin in other systems (38–40), our data support the primary importance of the CD14 pathway in mediating the response of alveolar macrophages to LPS. Previous studies showed that antibodies to CD14 prevent the binding of LPS/LBP bearing particles to the surface of monocyte-derived macrophages (5) and block the production of TNFα in whole blood in response to LPS (6). Our observation that an antibody directed at CD14 inhibits the response to LPS as well as LPS/LBP strengthens the interpretation that CD14 functions as an important part of a membrane complex that mediates the effects of LPS as well as LPS/LBP complexes.

The signals that follow binding of LPS/LBP complexes to CD14 remain uncertain. CD14 is anchored in the plasma membrane by a phosphatidylinositol tail (41). Phosphatidylinositol-anchored proteins are not typical G protein–linked receptors and it remains uncertain whether receptors such as CD14 transmit intracellular signals directly (42). An alternative possibility for CD14-mediated triggering of macrophages is that the binding of LPS/LBP complexes to CD14 does not transmit a signal directly, but regulates the density or affinity of a nearby second receptor that in turn transmits a signal intracellularly. Recent evidence provides indirect support for this possibility, as ligation of CD14 upregulates adherence receptors on monocytes causing aggregation (43). We found that neither LPS nor LPS/LBP complexes affect the initial concentrations of calcium of pH in rabbit alveolar macrophages, suggesting that these pathways are unlikely to contribute to intracellular signaling initiated by ligation of CD14 by LPS/LBP complexes. The effects of LPS/LBP complexes and CD14 on other signaling pathways associated with activation in macrophages, such as protein kinase C activation (44, 45) and tyrosine phosphorylation (46, 47), warrant further study.

We found that one important intracellular consequence of the signaling events initiated by LPS/LBP complexes is an increase in the kinetics of TNFα mRNA message expression. In alveolar macrophages treated with LPS and LBP, TNFα mRNA appeared earlier, accumulated to higher levels, and then disappeared earlier than in macrophages treated with LPS alone. The regulation of TNFα expression is complex, and depends on promoter sequences in the TNFα gene, AU rich sequences in the 3′-untranslated region of the TNFα mRNA that both destabilize the mRNA and repress its translation (48, 49), and ribonuclease activity that destroys the mRNA (50). The earlier appearance of TNFα mRNA that we observed suggests that LBP/LPS complexes increase the rate of transcription of the TNFα gene above that seen with LPS alone, although further direct analysis of gene transcription is necessary to prove this. The observation that TNFα mRNA half-life is prolonged by LPS/LBP complexes is supported by recent experiments with elicited peritoneal macrophages (51). The data do not establish whether the apparent increase in TNFα mRNA $t_{1/2}$ is a unique effect of LBP or a consequence of increased intracellular delivery of LPS in the presence of LBP.

Although these studies indicate that LBP enhances LPS-driven TNFα production by normal alveolar macrophages, it remains to be determined whether the effect of LBP is similar for other cytokines produced by alveolar macrophages. In other studies, we have found that LBP also accelerates the production of IL-8 by rabbit alveolar macrophages and the human monocytic cell line, THP-1. Furthermore, the effects of LBP on immunologically activated alveolar macrophages need to be studied in detail. Because TNFα is an important mediator of the sepsis syndrome that both causes and complicates lung injury, the finding that LBP accumulates in the lung during inflammation and regulates the production of TNFα by alveolar macrophages is likely to have clinical importance. Strategies to block the effects of LBP on alveolar macrophages may be useful in limiting some types of inflammatory reactions in the lungs.

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