Lipopolysaccharide Binding Protein and CD14 Interaction Induces Tumor Necrosis Factor-α Generation and Neutrophil Sequestration in Lungs After Intratracheal Endotoxin

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It has been proposed that lipopolysaccharide (LPS) bound to the 60-kD LPS binding protein (LBP) forms an LPS/LBP complex that, in turn, binds to the CD14 receptor on monocytes/macrophages and stimulates the release of cytokines. We examined the role of LBP and CD14 in tumor necrosis factor-α (TNF-α) production and neutrophil (polymorphonuclear leukocyte [PMN]) sequestration in lungs induced by intratracheal instillation of LPS using rabbit lungs perfused at constant flow with lactated Ringer-albumin solution. LPS alone (Salmonella minnesota, wild type; 20 ng) or in the presence of LBP (500 ng) was injected intratracheally. In some experiments, human PMNs (5×10⁶) were added to the perfusate after a 2-hour period of perfusion. Samples of lung perfusate were collected every 30 minutes for 180 minutes when bronchoalveolar lavage was also performed. TNF-α concentrations in the perfusate and bronchoalveolar lavage fluid were determined by use of a bioassay with L-929 fibroblasts, and PMN accumulation in lungs was determined by myeloperoxidase assay of lung homogenates. LPS alone did not significantly increase TNF-α production or lung PMN accumulation, whereas the LPS/LBP complex increased TNF-α concentration in perfusate two-fold and PMN accumulation two-fold compared with the effect of LPS alone. Intratracheal instillation of anti-CD14 monoclonal antibody MY4 (40 μg) with the LPS/LBP complex prevented TNF-α release and PMN sequestration, whereas an isotype-matched control monoclonal antibody was ineffective. Therefore, LBP in the airspace enhances the LPS effect on TNF-α production via a CD14-dependent pathway, and as a result, CD14 activation can contribute to lung PMN sequestration. Airspace accumulation of LBP secondary to increased vascular and airway epithelial injury may play a critical role in development of acute lung injury by promoting TNF-α production via a CD14-dependent mechanism. (Circulation Research 1993;73:15-23)

Key Words • lipopolysaccharides • endotoxemia • lipopolysaccharide binding protein • CD14 • acute lung injury • rabbit lungs

Intratracheal instillation of endotoxin, a lipopolysaccharide (LPS) component of gram-negative bacteria, causes acute lung injury.1 Both alveolar macrophages and polymorphonuclear leukocytes (PMNs) play a role in the LPS-induced lung inflammation. LPS stimulates macrophages to release inflammatory mediators such as arachidonic acid metabolites,2,3 oxygen radicals,4 and cytokines.5,6 Some of these mediators can directly mediate tissue injury, and they are also capable of recruiting PMNs to inflammatory sites, thereby augmenting the production of proinflammatory products.7-8 LPS-induced release of cytokines (ie, tumor necrosis factor [TNF] and interleukin-1) from alveolar macrophages increases the adhesivity of endothelial cells to PMNs by expression of cell-surface adhesion molecules such as intercellular adhesion molecule (ICAM-1) and endothelial adhesion molecule (E-selectin).9,10 In addition, chemoattractants (ie, interleukin-8 and leukotriene B₄) released from alveolar macrophages stimulated by LPS may induce PMN migration to inflammatory sites.5,11 Recent studies have proposed a new concept concerning the mechanism of LPS-induced stimulation of macrophages/monocytes, which may have implications in the pathogenesis of acute lung injury. LPS can complex with a 60-kD acute-phase serum protein, the LPS-binding protein (LBP), and the complex, in turn, binds to the CD14 receptors on monocytes/macrophages, resulting in activation of cells and release of cytokines and other humoral mediators.12,13 Little is known about the role of this pathway in vivo. Since sepsis is associated with increases in the permeability of vascular endothelial and airway barriers in lungs, serum proteins such as LBP (molecular mass, 60 kD) and albumin (molecular mass, 69 kD) can accumulate in the airspace.14 The airspace LBP may enhance the effects of LPS on alveolar macrophages in the production of

Received February 22, 1993; accepted April 2, 1993.

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cytokines. The activation of this pathway mediated by formation of the LPS/LBP complex may play an important role in the progression of lung injury. In the present study, we examined whether purified LBP in the airspace of rabbit lungs induces TNF-α production after LPS challenge and, in turn, mediates lung PMN sequestration. We also determined the contribution of the CD14 receptor in the response by using an anti-human CD14 monoclonal antibody that cross-reacts with rabbit CD14.

Materials and Methods

Materials

Pyrogen-free sterile lactated Ringer’s solution, sterile 0.9% saline, and sterile water were obtained from Baxter-Travenol, Deerfield, Ill. Human serum albumin (25%) was obtained from the American Red Cross, Albany, NY. Gentamicin sulfate and RPMI-1640 containing streptomycin and penicillin were purchased from Whittaker Bioproducts, Walkersville, Md. LPS (Salmonella minnesota, wild type) was obtained from List Biological Laboratories, Inc, Campbell, Calif. The anti-CD14 monoclonal antibodies (MY4 and FMC32) were obtained from the Fourth Leukocyte Typing Workshop, Vienna, 1989; both are of the immunoglobulin (Ig) G3 isotype. Fluorescein isothiocyanate–conjugated F(ab')2 fragments of a goat anti-mouse IgG antibody were purchased from Cappel Laboratories, Durham, NC.

Cell Separation

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized rabbit blood by density-gradient centrifugation using Lymphopaque (Accurate Chemical & Scientific Corp, Westbury, NY) as described. The PBMC layer was collected, and the cells were washed twice in RPMI-HEPES (GIBCO-BRL, Gaithersburg, Md).

Human PMNs were isolated from the blood of healthy volunteers by a one-step separation using neutrophil isolation medium (Cardinal Associates, Santa Fe, NM). The purity of PMNs evaluated by Giemsa staining was >99%, and availability determined by trypan blue exclusion was >98%.

Fluorescence Analysis

PBMCs were stained indirectly with the anti-CD14 monoclonal antibodies (mAbs) and analyzed on a flow cytometer as described. Briefly, purified MY4, FMC32, or a nonspecific mouse IgG3 isotype control (Sigma Chemical Co, St Louis, Mo) was resuspended at a concentration of 2.5, 5.0, or 10.0 μg/mL, respectively, in a diluting buffer consisting of phosphate-buffered saline (PBS) containing 1% albumin and 0.02% sodium azide. For staining, PBMCs were washed twice with the diluting buffer and incubated with aggregated human IgG (5 minutes at room temperature) to block Fe-receptor binding. Diluted anti-CD14 antibodies or the isotype control was added (50 μL/5×10^6 cells), and the cells were incubated at 4°C for 30 minutes. The cells were washed three times with the diluting buffer; fluorescein isothiocyanate–conjugated anti-mouse IgG (pretitered) was added (50 μL); and the cells were incubated as above. Finally, the cells were washed two times as described above, resuspended in 300 μL diluting buffer, and analyzed for fluorescence on a Coulter Profile cytometer (Coulter Immunology, Hialeah, Fla). Analysis was performed using Profile software.

Preparation of LPS

LPS extracted from Salmonella minnesota (wild type, List Biological Laboratories) was prepared by suspending 10 mg in 2 mL sterile water and sonicating until clarified (three to five times in 20-second bursts at maximum intensity using a sonicator). Aliquots of the LPS stocks were stored at −80°C. All LPS working dilutions were prepared in sterile saline.

Purification of LBP and Formation of Complexes With LPS

LBP was purified from rabbit acute-phase serum by ion-exchange chromatography. Briefly, 50 mL hydrated Bio-Rex 70 resin (Bio-Rad Laboratories, Richmond, Calif) was equilibrated with 41 mM NaCl in 50 mM phosphate buffer, pH 7.3, containing 2 mM EDTA (PBE). Acute-phase serum (180 mL, containing 5 mM EDTA) taken 24 hours after subcutaneous administration of 1 mL of 3% silver nitrate was applied to the column. The column was extensively washed with equilibration buffer until the optical density at 280 nm (OD_280) reached 0, followed by a similar wash with 220 mM NaCl in PBE. Elution was performed with a linear gradient from 220 to 500 mM NaCl in PBE. Six fractions (pooled according to OD_280) were dialyzed against saline and assayed for their ability to bind to radiolabeled LPS as previously described. Briefly, 1 mL of each fraction was mixed with 10 μg of 14C biosynthetically labeled LPS from Salmonella typhimurium PR122 (List Biological Laboratories), incubated at 37°C for 30 minutes, mixed with 10.5 mL of a solution of CsCl (2.49 M in 0.15 M NaCl), and subjected to density gradient centrifugation (277 000g in a Ti70 rotor [Sorvall Instruments, Wilmington, Del]) for 18 hours at 4°C. After centrifugation, the gradients were fractionated in 200-μL aliquots and counted for radioactivity in a scintillation counter. Two fractions elicited a radioactive peak at 1.3 g/mL and were suspected to contain LBPs. All fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) as previously described and were stained directly with Coomassie blue. For the instillation into airways, the mixture of LPS (20 ng) and purified LBP (500 ng) (final volume, 1 mL) was preincubated for 30 minutes at 37°C before airway instillation.

Cell Activation

For the cell activation assays, the LPS/LBP complex was formed by incubating various amounts of LPS with 60 ng purified rabbit LBP diluted in RPMI-HEPES for 30 minutes at 37°C as previously described. PBMCs (8×10^7) were resuspended in 400 μL RPMI-HEPES and incubated with purified antibodies (5 μg/mL) at 4°C for 10 minutes. LPS/LBP complex or LPS alone was then added to the antibody-treated PBMCs, and the cells were incubated for 3.5 hours at 37°C in 5% CO_2. The cell-free supernatant was assayed for TNF-α.
**Lung Perfusion**

New Zealand White rabbits (1.8 to 2.2 kg, Franklin’s Rabbity, Raleigh, NC) were anesthetized with an intramuscular injection of xylazine (5 mg/kg, Moby Corp, Shawnee, Kan.), acepromazine maleate (0.75 mg/kg, Aveco, Fort Dodge, Iowa), and ketamine (35 mg/kg, Parke-Davis, Morris Plains, NJ). The trachea was cannulated before opening the thorax. Sodium pentobarbital (25 mg/kg, Fort Dodge Laboratories, Fort Dodge, Iowa) and sodium heparin (700 U/kg) were administered intravenously, and the animals were exsanguinated by severing the abdominal aorta. The heart and lungs were removed en bloc and suspended from one end of a beam balance. The lung perfusion system used in these experiments has been described previously.²¹ Perfusion catheters were placed in the pulmonary artery and left atrium. Pulmonary artery pressure, left atrium pressure, and lung wet weight were continuously monitored during the experiment. The lungs were inflated at 20 cm H₂O pressure several times to reverse atelectasis. After these brief inflations, the airway pressure was maintained at 1 cm Hg with 95% O₂–5% CO₂ during the experimental period. Perfusion of the system was begun within 10 minutes of pneumothorax using a peristaltic pump (model 1215, Harvard Apparatus, Millis, Mass). The perfusate consisted of phosphate-buffered lactated Ringer’s solution containing 0.5% human serum albumin and the following components (mM): NaCl, 100; KCl, 4; CaCl₂, 1.4; sodium lactate, 28; NaHCO₃, 0.06; NaHPO₄, 0.13; and Na₂HPO₄, 0.869. The perfusate was warmed to maintain a constant temperature of 37°C, and the perfusion rate was set at 60 mL/min for all experiments. At the beginning of the perfusion, 600 mL of buffer was perfused through the system and discarded to remove any residual plasma and circulating blood cell marginated in the pulmonary circulation. The final perfusate volume was 600 mL. The monitored parameters were stable in control lungs perfused for up to 3 hours, although experimental perfusions were always <3 hours. There were no significant differences in pulmonary artery and left atrium pressures and changes in lung weight among the different treatment groups.

**Maintenance of Endotoxin-Free Conditions**

The endotoxin concentration of the perfusate and reagents in these experiments was measured using the limulus lysate assay (E-Toxate, Sigma). We were able to maintain the perfusate essentially endotoxin free (<100 pg/mL) for the 3-hour duration of the experiment. The perfusion buffer was pyrogen free and sterile, and sterile lactated Ringer’s solution and other reagents were used for injections. Gentamicin sulfate (25 mg/L) was added to the buffer to prevent bacterial growth during the experiment. All disposable equipment used in the perfusion circuit was sterile and pyrogen free. Non-disposable equipment was washed in laboratory detergent, then washed in E-Toxaclean (Sigma), and rinsed with endotoxin-free deionized water before use.

**Experimental Lung Protocols**

Either LPS alone (20 ng/mL) (n=5), LBP alone (500 ng/mL) (n=5), or LPS/LBP complex (LPS [20 ng]+rabbit LBP [500 ng]) in a total volume of 1 mL, precultivated for 30 minutes at 37°C (n=5) was instilled intratracheally into the Ringer-albumin-perfused lungs. These agents were instilled in two 500-μL saline injections via the tracheal cannula, followed by full inflation of the lungs at 20 cm H₂O airway pressure for five breaths. This procedure distributed the solution to the peripheral lung, as ascertained visually using a trypan blue dye solution. In a control group (n=5), an equal volume of saline was instilled. The pulmonary hemodynamic parameters and weight gain were monitored during the experiment. Samples of the lung perfusate were collected every 30 minutes for a total of 180 minutes. In some experiments (n=5), after 120 minutes of perfusion, freshly isolated human PMNs (5×10⁶ cells) were instilled into the pulmonary artery catheter within 5 minutes and allowed to circulate for the remaining 60 minutes. In another group of experiments, the cross-reacting anti-CD14 mAb MY4 (40 μg/mL) (n=5) was instilled to study the role of the CD14 receptor. Anti-CD14 mAb FMC32 (40 μg/mL) (n=4) was used as a control nonneutralizing antibody (see “Results”). At the end of the 180-minute period of perfusion, the right lung was lavaged using two aliquots of LPS-free saline (15 mL). The left lung was kept in a low-temperature freezer (−70°C) for the subsequent assay for lung tissue myeloperoxidase activity (see below).

**Collection of BAL Fluid and Alveolar Macrophages**

Bronchoalveolar lavage (BAL) fluid was centrifuged at 400g for 7 minutes, and the supernatant was frozen at −70°C before TNF-α assay. The pelleted cells were resuspended in RPMI-1640 supplemented with 100 U/mL penicillin and 100 ng/mL streptomycin and washed two times. Cell differentials and percent viability counts were determined. The cells were plated in a 24-well plate at a concentration of 10⁶ cells/mL, allowed to adhere for 60 minutes at 37°C in 5% CO₂, and washed two times with RPMI-1640 to remove any nonadherent cells. The adherent cells were maintained for 3 hours in serum-free RPMI under the same conditions as above, after which the supernatants were collected and assayed for TNF-α activity.

**TNF-α Assay**

TNF-α activity was determined by a cytolytic assay using L929 murine fibroblasts as modified from a procedure described by Kirstein et al.²² In brief, L929 fibroblasts (gift of Dr Corrado Baglioni, Department of Biological Sciences, State University of New York at Albany) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. The cells were plated into a 24-well culture plate at a density of 2×10⁴ cells per well and incubated in 5% CO₂ at 37°C until they became confluent (within 22 to 24 hours). The cells were washed with fresh medium, and the medium containing 10 μg/mL cycloheximide was added. Serially diluted samples or TNF-α standard (Cetus Laboratories, Palo Alto, Calif) was added to the medium to give a final volume of 1 mL. After incubation for 22 to 24 hours in 5% CO₂ at 37°C, the medium was removed, and the cells were washed two times with Hanks’ balanced salt solution. Cell lysis was detected using neutral red uptake by incubating with 0.25 mL neutral red solution (0.004%) in PBS for 1 hour at 37°C. The cells were fixed with 0.5 mL formaldehyde (4%)
calcium chloride (1%) solution for 45 seconds, and the dye was dissolved in 0.5 mL ethanol (50%)/acetic acid (1%). Aliquots were transferred to a 96-well microtiter plate and read at 550-nm using a microplate reader (MR 600, Dynatech Laboratories, Inc, Chantilly, Va). Cytotoxic activity associated with TNF-α was confirmed by inhibiting the response with a cross-reacting goat anti-TNF-α polyclonal antibody (gift of Dr John C. Mathison, Scripps Research Institute, La Jolla, Calif). Specific activity was determined from a standard curve obtained with recombinant human TNF-α.

**Lung Myeloperoxidase Assay**

Lung myeloperoxidase (MPO) activity was assayed to quantitate pulmonary neutrophil uptake. MPO was extracted from left lung tissue, and the content was measured as modified from the method of Goldblum et al. The lung tissue was suspended in hexadecyltrimethylammonium bromide (HTAB, Sigma) at 5 mL/g tissue in 50 mM phosphate buffer adjusted to pH 6.0 and homogenized on ice for 30 seconds with a Polytron. The homogenate was centrifuged at 40 000g for 10 minutes. The initial supernatant was then discarded. The pellet was resuspended in HTAB. The sample was freeze-thawed (for 20 minutes at -70°C), followed by homogenation and centrifugation. The supernatant MPO activity was assayed by mixing a 0.1-mL aliquot of the sample with 2.9 mL of 50 mM PBS containing 0.0005% hydrogen peroxide and 0.167 mg/mL o-dianisidine dihydrochloride (Sigma). The change in absorbance was measured at 460 nm for 3 minutes using a DU spectrophotometer (Beckman Instruments, Inc, Fullerton, Calif). The MPO activity was expressed as the change in absorbance per minute per gram of tissue.

**Statistical Analysis**

The data were analyzed by two-way analysis of variance. Comparisons between groups were made using Student's t test with a Bonferroni correction for multiple comparisons. Data are presented as mean±SEM. Statistical significance was set at P<.05.

**Results**

**Binding of Anti-Human CD14 mAbs MY4 and FMC32 to Rabbit CD14**

Rabbit PBMCs were analyzed for their ability to bind anti-human CD14 mAbs. Indirect immunofluorescent staining of cells with two anti-human CD14 mAbs (MY4 and FMC32) or an isotype control (IgG₂) showed specific binding of both MY4 and FMC32 to the monocyte population (mean channel fluorescence, 5.244, 1.586, and 0.392, respectively) and no binding to the lymphocyte population (mean channel fluorescence, 0.213, 0.227 and 0.239, respectively) (Fig 1). Both mAbs also detected CD14 on rabbit PMNs (data not shown). The specificity of mAbs MY4 and FMC32 for monocytes suggests that they detect the CD14 antigen on rabbit monocytes.

**Purification of Rabbit LBP**

LBP was purified from rabbit acute-phase serum as described in "Materials and Methods," and fractions were assayed for LBP activity. As shown in Fig 2A, fraction 3 was able to bind [14C]LPS; the [14C]LPS/LBP complex had a density of 1.3 g/mL as determined by CsCl isopycnic density gradient ultracentrifugation. The profile of fraction 3 analyzed by SDS-PAGE is shown in Fig 2B. Two bands (a primary band of 60 kD and a minor band of 58 kD), detected after Coomassie blue and silver staining (data not shown), were similar in size to those previously described for LBP and shown to have identical N-terminal sequences.16

**Inhibition of LPS-Induced Activation of Rabbit Monocytes With mAb MY4**

Previous studies have shown that anti-CD14 antibodies inhibit the TNF-α generation from human monocytes and neutrophils in response to LPS/LBP complex challenge. To determine whether MY4 or FMC32 mAbs could inhibit the activation of rabbit monocytes by the LPS/LBP complex, we studied the effects on rabbit monocyte TNF-α release. As shown in Fig 3,
rabbit PBMCs pretreated with mAb MY4 and incubated with LPS/LBP secreted fivefold less TNF-α than did untreated PBMCs (12 U/mL versus 56 U/mL). In contrast, treatment of rabbit PBMCs with mAb FMC32, which also cross-reacts with rabbit CD14 and is of the same isotype as MY4 but is nonneutralizing, had no significant effect on TNF-α release. These results show that mAb MY4 specifically inhibits the LPS/LBP complex–induced activation of rabbit PBMCs.

**TNF-α Generation in Perfusate After Intratracheal LBP/LPS Challenge**

After intratracheal instillation of LPS alone (20 ng) into the Ringer-albumin–perfused lungs, the TNF-α concentration in the perfusate increased slightly and reached a peak of 962±484 pg/mL at 180 minutes (not significantly different compared with the control value) (Fig 4). The intratracheal instillation with saline alone or LBP alone showed minimal increases in TNF-α concentrations (ie, 182±90 and 158±110 pg/mL at 180 minutes, respectively). In contrast, intratracheal instillation of LPS/LBP complexes showed a marked increase in the perfusate TNF-α concentrations. The increase occurred at 150 minutes (P<.05) and reached a value of 3560±521 pg/mL (approximately threefold greater than LPS alone) at 180 minutes (Fig 4).

In some experiments, PMNs were added to the perfusate at 120 minutes. No significant differences in the time course of TNF-α release or the maximum

**Fig 3.** Graph showing effects of anti-human CD14 monoclonal antibodies on the lipopolysaccharide (LPS) activation of rabbit peripheral blood monocytes. Rabbit monocytes (8×10⁶) were treated with the purified anti-CD14 monoclonal antibodies (Mabs) MY4 or FMC32 at a final concentration of 5 μg/mL or with no Mab. Various amounts of LPS complexed to 60 ng rabbit LPS binding protein were added, and after a 3.5-hour incubation at 37°C in 5% CO₂, the supernatants were assayed for tumor necrosis factor-α (TNFα). Data shown are representative of two independent experiments.

**Fig 4.** Graph showing tumor necrosis factor-α (TNFα) production in lung effluent in rabbit lungs perfused with Ringer-albumin solution (see "Materials and Methods"). LPS, lipopolysaccharide; LBP, LPS binding protein; PMN, polymorphonuclear leukocytes; I.T., intratracheal. The following conditions were tested: (1) I.T. instillation of 0.9% NaCl (control), (2) I.T. LPS (Salmonella minnesota, wild type; 20 ng), (3) I.T. LPS/LBP complex (LPS [20 ng]+rabbit LBP [500 ng], preincubated for 30 minutes at 37°C), (4) I.T. LPS/LBP without PMN (5×10⁶) added to the perfusate 120 minutes after the initiation of the perfusion. Samples of the lung perfusate were collected every 30 minutes for 3 hours for subsequent TNFα analysis. Bars indicate ±SEM. *Different from earlier times and other groups at the corresponding times (P<.05).
TNF-α concentration in the perfusate were evident between the two groups with or without PMNs (Fig 4), indicating that any residual margined PMNs were not the source of TNF-α released into the perfusate.

**TNF-α Generation in BAL Fluid**

The Ringer-albumin-perfused lungs were lavaged after the 180-minute perfusion period, and the TNF-α concentration in BAL fluid was determined. Minimal alterations in TNF-α were detected in the lavage fluid obtained at 180 minutes after intratracheal LPS challenge and in the control group intratracheally challenged with saline (Fig 5). The LBP group also showed no significant increase in TNF-α concentration in the lung perfusate (data not shown). However, TNF-α concentration in BAL fluid increased significantly (P<.05) only after the intratracheal instillation of LPS/LBP complex (Fig 5).

**TNF-α Generation by Alveolar Macrophages Isolated After Intratracheal LPS/LBP Challenge**

We measured the TNF-α production by alveolar macrophages obtained from lungs intratracheally challenged with LPS, LBP, or LPS/LBP complex in situ. The isolated macrophages were incubated for a further 3 hours in serum-free RPMI, and culture supernatants were assayed for TNF-α activity. Alveolar macrophages obtained from LPS/LBP complex–challenged lungs produced greater amounts of TNF-α than did macrophages obtained from the LPS-challenged group (1418±622 pg/10⁶ cells versus 196±103 pg/10⁶ cells, respectively) (Fig 6), suggesting that alveolar macrophages were an important source of TNF-α generation after the in situ LPS/LBP challenge. Intratracheal instillation of LBP alone showed no significant difference in TNF-α production as compared with saline control.

**Neutralizing Anti-CD14 mAb Prevents TNF-α Generation**

We determined the effects of the cross-reacting anti-CD14 mAbs MY4 (shown above to inhibit rabbit monocyte LPS/LBP–induced activation in vitro) and FMC32 (shown above to react with rabbit monocytes but to have no effect on activation) to study the role of the CD14 receptor on macrophages/monocytes in the LPS/LBP–induced TNF-α production from rabbit macrophages. Either mAb MY4 or mAb FMC32 (40 μg in 1 mL saline) was instilled intratracheally with the LBP/LPS complex. mAb MY4 prevented the TNF-α generation induced by the LPS/LBP complex (P<.05), whereas the control mAb FMC32 showed no inhibitory effect (Fig 7). The BAL fluid obtained from mAb MY4–treated lungs also showed a reduction in TNF-α concentration.
as compared with mAb FMC32–treated lungs (Fig 5). This was also the case with alveolar macrophages harvested from the MY4- and FMC32–treated lungs; the former showed negligible TNF-α production as compared with macrophages obtained from FMC32–treated lungs (Fig 6). Intratracheal challenge with mAb MY4 or FMC32 alone did not have any significant effect on TNF-α generation.

**Lung PMN Accumulation Induced by Intratracheal Challenge of LBP/LPS**

PMNs were added to the perfusate at 120 minutes after LPS challenge to determine whether the TNF-α release contributed to PMN uptake in the lungs. PMN accumulation in the lung at 180 minutes was determined by an MPO assay of lung homogenates. As shown in Fig 8, MPO activity in lungs challenged with LPS alone increased slightly, whereas challenge with LPS/LBP complex showed a greater increase of MPO activity (P < .05). Intratracheal instillation of mAb MY4 with LPS/LBP challenge significantly reduced the increase of MPO activity (P < .05), whereas control mAb FMC32 showed no inhibitory effect (Fig 8).

**Discussion**

Previous in vitro studies have shown that LPS can activate macrophages (including alveolar macrophages) to produce TNF-α. LBP, an acute-phase plasma protein, can greatly enhance the sensitivity of this response by forming a complex with LPS. The LBP/LPS complex binds to the CD14 receptor on the surface of macrophages/monocytes, which in turn can augment the production of TNF-α even at low LPS concentrations (<20 ng/mL). It is believed that LBP facilitates macrophage/monocyte activation in response to LPS via an LBP/CD14–dependent pathway. Recently, Martin et al have reported that immunoreactive LBP is nominally present in very low concentrations (≈40 ng/mL) in the alveolar fluid and that its concentration increases up to 120 to 600 ng/mL in patients with acute respiratory distress syndrome. The accumulation of LBP/LPS in the airspace and the complexing of LBP with LPS may stimulate the production of cytokines such as TNF-α by alveolar macrophages. Such an accumulation of LBP/LPS in the airspace secondary to endothelial and airway epithelial injury associated with sepsis might play a role in the progression of lung injury. This process may be associated with localized extravasation of plasma protein. In the present study, we examined whether airway LPS/LBP complex was a sufficient stimulus to induce the production of TNF-α and whether TNF-α was detectable in the pulmonary circulation, with its potential consequence of mediating neutrophil sequestration.

We show that 20 ng LPS administered intratracheally into the rabbit lungs perfused with Ringer-albumin solution did not result in TNF-α generation in the lung perfusate; however, intratracheal instillation of the LPS/LBP complex (in which the amount of LPS was also 20 ng) markedly increased the TNF-α concentration in the perfusate. This effect of the LPS/LBP complex was prevented by intratracheal injection of a rabbit cross-reacting anti-C14 mAb MY4, demonstrating that airway LBP mediated the TNF-α generation via a C14–dependent pathway. In contrast, the control isotype-matched mAb FMC32, which did not prevent TNF-α production in isolated rabbit monocytes in response to LBP/LPS challenge, failed to prevent TNF-α generation in the rabbit lung. The delayed nature of TNF-α generation (requiring =2 hours after intratracheal instillation of the LPS/LBP complex) is consistent with the time required for de novo synthesis of TNF-α after CD14 activation.

There are several different cell types in lungs that could respond to LPS/LBP complex stimulation: alveolar macrophages, lung tissue macrophages, and residual leukocytes, including monocytes and neutrophils (PMNs) that can remain adherent to the surface of endothelial cells (ie, those cells not washed away by the perfusate) or cells that were marginalized in the microvasculature. Although PMNs can release TNF-α when challenged with the LPS/LBP complex, they are an unlikely source, since addition of PMNs to the perfusate did not augment the TNF-α production. This may be related to the lower level of CD14 expressed on PMNs. In the present study, it is most likely that monocytes/macrophages are the important source of TNF-α production in the lung perfusate. LPS/LBP is known to stimulate the production of TNF-α by alveolar macrophages. Since the generated TNF-α can increase the permeability of the epithelial and vascular endothelial barriers and since intratracheally administered TNF-α can rapidly "leak" into the pulmonary circulation by crossing the epithelial barrier, TNF-α measured in the perfusate may be the result of macrophage activation and subsequent increased permeability of the airway epithelial barrier. The conditioned medium of macrophages obtained from the LBP/LPS–challenged lungs showed higher levels of TNF-α than the macrophage-conditioned medium obtained from either LPS- or LBP-challenged lungs, suggesting a role for alveolar macrophages as the source of TNF-α in the perfusate after intratracheal instillation of the LBP/LPS complex. Another possible mechanism of TNF-α generation in the
perfusionate may be via penetration of LPS and LBP into the vascular space, such that the LBP/LPS complex is able to stimulate the marginated or adherent blood monocytes/macrophages and release TNF-α directly into the circulation; however, this is improbable because the high molecular mass of LBP (60 kD) would markedly restrict its passage across epithelial and endothelial barriers.

TNF-α induces sequestration of PMNs in the pulmonary vascular bed. Intratracheal injection of TNF-α (8 μg) caused severe neutrophilic inflammatory exudate beginning at 6 hours. In the present study, we observed that TNF-α released in the lung effluent was coupled to PMN sequestration in lungs, as determined by the increase in lung tissue MPO content, suggesting that TNF-α release was a factor contributing to PMN accumulation. The TNF-α-mediated upregulation of ICAM-1 and E-selectin on the surface of endothelial cells may lead to PMN adhesion and lung PMN sequestration. Both adhesion molecules are expressed within a 2-hour time frame. In addition, macrophages can produce other proinflammatory cytokines such as interleukin-1, interleukin-6, which may also contribute to PMN accumulation.

We have shown that increased adhesiveness of PMNs to endothelium produced by TNF-α is a critical factor mediating PMN-dependent vascular endothelial injury. The present data suggest that the production of TNF-α by alveolar macrophages after stimulation with the LPS/LBP complex and the subsequent "leakage" of TNF-α into the vasculature contribute to PMN sequestration. If the sequestered PMNs are activated, they are capable of injuring the vascular endothelial barrier.

In conclusion, LBP's in the airway markedly augmented the effect of LPS on TNF-α production via a CD14-dependent pathway. TNF-α (and possibly other inflammatory cytokines) released by the activated macrophages into the circulation lead to PMN accumulation, which may be a critical step in the progression of lung injury associated with sepsis.

Acknowledgments

This study was supported by National Institutes of Health grants HL-27016, HL-45638, and HL-46350 (Dr Malik); and Tobacco Research Council grant 2218 and National Institutes of Health grant A123859 (Dr Goyert). Dr Goyert is a Leukemia Society of America Scholar, and Dr Haziot is an American Heart Association Fellow.

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Circ Res. 1993;73:15-23
doi: 10.1161/01.RES.73.1.15
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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