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CD14 and Tissue Factor Expression by Bacterial Lipopolysaccharide-Stimulated Bovine Alveolar Macrophages In Vitro

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The membrane-associated CD14 receptor (mCD14) is a monocyte/macrophage differentiation antigen, and it has been demonstrated to serve as a receptor for bacterial lipopolysaccharide (LPS; endotoxin). Binding of LPS to mCD14 has been shown to be associated with LPS-induced macrophage, monocyte, and neutrophil activation in humans. In this report, we describe the presence and function of an mCD14-like receptor on bovine alveolar macrophages (bAM). An immunofluorescence technique and flow cytometric analysis indicated binding of anti-human CD14 monoclonal antibodies (MAb) My4, 3C10, and 60bd to bAM. Binding of anti-CD14 MAb (3C10 and MY4) was reduced over 20% by pretreatment of bAM with phosphatidylinositol-specific phospholipase C (0.5 to 1.0 U/ml), indicating that bovine mCD14 is a glycosyl phosphatidylinositol-anchored protein. In addition, pretreatment of bAM with anti-CD14 MAb decreased binding of ^{125}I-labeled LPS to macrophages, suggesting that bovine mCD14 serves as a receptor for LPS. A cDNA probe based on the human sequence for CD14 was used in Northern (RNA) blot analysis, and hybridization to human monocyte CD14 yielded the expected 1.5-kb band. Hybridization to bovine mRNA yielded a 1.5-kb band plus an unexpected 3.1-kb band. Constitutive expression of bovine CD14 mRNA was observed, and the expression level was modestly elevated in bAM stimulated for 24 h with LPS (1 ng/ml) in the presence of bovine serum. The function and activation of bAM were assessed by quantitation of tissue factor (TF) expression on the cells using an activated factor X-related chromogenic assay and S-2222 substrate. LPS (1 ng/ml)-mediated upregulation of TF expression on bAM was dependent on the presence of bovine serum components, and TF expression was inhibited by anti-CD14 MAb. In addition, TF mRNA levels in LPS-stimulated bAM were decreased by pretreatment of cells with anti-CD14 MAb (MAb 60bd, 10 μg/ml).

Bacterial lipopolysaccharide (LPS) is a potent toxin released from gram-negative bacterial cell walls, and it is notorious for initiation of inflammation and medical dilemmas (28). Cells of macrophage lineage are exquisitely sensitive to the presence of LPS, and when activated by the presence of this bacterial product, they may orchestrate a multitude of proinflammatory processes (8, 42). In the lungs, alveolar macrophages play prominent regulatory roles in host defense, but in some circumstances the macrophages may initiate edema formation, excessive leukocyte infiltration, and fibrin deposition in alveolar air spaces in response to the presence of gram-negative bacteria and LPS (46, 47).

Factors involved in the interaction of LPS with myelomonocytic cells have recently been elucidated. LPS-binding protein (LBP) is a 58- to 60-kDa glycoprotein present in the sera of humans (32), rabbits (40), and mice (13), and it is an acute-phase protein which increases in concentration in response to inflammatory stimuli. LBP in serum binds LPS with high affinity (32, 41), and the resultant LBP-LPS complex binds to mCD14 receptors (31, 44, 49) on the surfaces of monocytes, macrophages, and neutrophils. The 53- to 55-kDa membrane-associated CD14 receptor molecule (mCD14) has been observed to be present on cells of human and murine origin, and this receptor possesses a glycosyl phosphatidylinositol anchor (16, 34). Other LPS receptors have also been described (22). A soluble form of CD14 is also normally present in human plasma (2), lacks the lipid tail, and has a slightly lower molecular mass (48 to 50 kDa) than its membrane-bound cousin. LBP has been found to shuttle LPS to the soluble form of CD14 (14), utilizing the amino-terminal half of LBP for LPS binding and the carboxyl-terminal half for interactions with CD14 (15). Interaction of LBP and mCD14 with LPS amplifies the response of macrophages to LPS, and it results in the generation of proinflammatory cytokines such as tumor necrosis factor alpha and interleukin 6 (10, 27, 32, 50). Formation of proinflammatory and immunomodulatory cytokines as a result of exposure to LPS may be beneficial to the host, but excessive cytokine synthesis may be detrimental.

The lung is a target for gram-negative bacterial infections in many species, including cattle, and interactions of alveolar macrophages with LPS are important in determining the outcome of these infections (9, 47). In research laboratories, many investigators utilize bovine sera or cells for in vitro studies, but published work has not clarified the factors involved in interactions of cells or fluids of bovine origin with LPS. A limited amount of bovine-related data exists, including reports that uncharacterized bovine serum factors bind LPS (19) and enhance association of LPS with peripheral-blood leukocytes (5). Bovine serum factors also promote activation of bovine macrophages in the presence of LPS (50). Our objective in the current study was to further characterize the factors involved in LPS-mediated activation of bovine alveolar macrophages (bAM). The results suggest the presence of mCD14 on bAM and indicate that bAM are dependent on an mCD14-like re-

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ceptor and LBP-like serum factors for LPS-mediated cellular activation and tissue factor expression. The findings suggest that the function of bovine mCD14 is similar to that reported for human mCD14.

MATERIALS AND METHODS

Alveolar macrophages. bAM were collected from 1- to 3-month-old Holstein-Frisian calves by volume-controlled bronchopulmonary lavage as has been described previously (21). Lavage fluid with cells was filtered through sterile gauze, and cells were pelleted by centrifugation, resuspended, and washed twice in sterile, pyrogen-free buffer before final resuspension in Dulbecco's modified Eagle's medium. The percent macrophages, determined with a Wright-Giemsa-stained cytocentrifuge preparation, was greater than 90%, and viability, determined by trypan blue dye exclusion, was also greater than 90%. The protocol for collection of alveolar macrophages was approved by an animal care committee at our institution, and animals serving as donors received the highest level of care.

Tissue factor assay. Tissue factor expression on the macrophages was quantitated by a colorimetric assay (35) based on digestion of substrate S-2222 by activated factor X. The assays were performed as previously described by our laboratory (50). Treatment variables included macrophages exposed to various concentrations of LPS (*Escherichia coli* O55:B5) alone or in combination with quantities of fetal bovine serum (FBS) or a bovine serum chromatography fraction (described below). In some assays monoclonal antibodies (MAb) or isotype-matched MAb of irrelevant specificity (control MAb) were added 20 min before addition of the LPS plus serum. We found the colorimetric assay for tissue factor to be very consistent with the modified prothrombin assay (6).

Bovine serum chromatography. Pooled bovine serum collected from normal, healthy cows was fractionated by using Bio-Rex 70 chromatography medium, previously described for use with rabbit serum (40) and with bovine serum (50). Fifty milliliters of Bio-Rex resin was equilibrated with 41 mM NaCl in 50 mM phosphate buffer, pH 7.3, containing 2 mM EDTA (phosphate-EDTA). Two hundred milliliters of bovine serum containing 5 mM EDTA was run over the column at 60 ml/h. The column was then washed with column equilibration buffer until the A_{280} of the eluate was <0.2 absorbance units. Washing with 220 mM NaCl in phosphate-EDTA was continued until the absorbance was <0.2, and then it was followed by washing with a linear gradient formed from 60 ml each of 220 and 500 mM NaCl in phosphate-EDTA. Finally, the column was washed with 1 M NaCl in phosphate-EDTA. Pools of fractions were dialyzed against 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.3) and concentrated approximately 10-fold by using YM10 membranes in an Amicon ultrafiltration cell. The bovine serum chromatography fraction used in our current work corresponds to the column eluate obtained when washing was done with 220 mM NaCl.

Immunofluorescence assay. bAM were first preincubated with murine immunoglobulin G (IgG) (20 to 100 μg/ml) in phosphate-buffered saline (PBS; pH 7.3) for 30 min to block nonspecific binding sites, and then anti-human CD14 antibodies (10 μg/ml) or another non-CD14 antibody known to bind to bovine macrophages was added. The latter was MAb 60.3, a mouse anti-human IgG2a that bound avidly to bAM in our own studies and which recognizes the CD18 cell surface glycoprotein complex (3). The specificity of MAb 60.3 for bovine neutrophils has been verified previously (4), and the presence of the CD18 complex on bovine leukocytes has been documented (33). After incubation at 37°C for 30 min, cells were washed twice with PBS and then incubated for 10 min with 5% goat serum to block nonspecific binding sites. Fluorescein isothiocyanate-labeled goat anti-mouse IgG (20 μg/ml) was then added for a 30-min incubation period. bAM were then washed twice and fixed with 0.5% paraformaldehyde. Fluorescence analysis was accomplished by utilizing a Becton Dickinson FACScan device with its analysis gate set to collect and quantitate the fluorescein isothiocyanate-antibody associated with bAM. Ten thousand cells were counted per treatment, and data were expressed as relative mean fluorescence intensities. In the experiments using phosphatidylinositol-specific phospholipase C (PI-PLC), bAM were preincubated with PI-PLC (0.01 to 1.0 U/ml) for 1 h at 37°C and washed twice with Hanks balanced salt solution, and then the antibody labeling procedure just described was performed.

Binding of I^{125}-labeled LPS to bAM. LPS from *E. coli* O111:B4 was sonicated, and then it was labeled by first being coupled to sulfosuccinimidyl-2-(*p*-azidosalicylamido)1,3′-dithiopropionate (ASD) as described previously (48). The LPS-ASD complex was radiolabeled with $Na^{125}I$ by using chloramine T (43, 48). The product (^{125}I-ASD-LPS) regularly had a specific activity of 6×10^9 cpm/mg of LPS. bAM were preincubated with anti-CD14 antibody 60bd (20 μg/ml) or an isotype-matched MAb of irrelevant specificity for 30 min and then incubated with ^{125}I-ASD-LPS with or without bovine serum for 1 h in the dark at 37°C in 5% CO_2 and ambient humidity. Bound ligand (^{125}I-ASD-LPS) was then subjected to photolysis for 10 min at room temperature by using short-wave UV irradiation (UVC 1000 cross-linker; Hoefer Scientific Instruments, San Francisco, Calif.; maximal emission at 254 nm). ^{125}I-ASD-LPS was linked (by photolysis) to molecules on the macrophages with which LPS became associated. Cells were subsequently fixed with 0.5% paraformaldehyde for 10 min and then pelleted by centrifugation and washed three times with PBS to remove unbound radioactivity. Macrophages were assayed for gamma emission as an indication of bound LPS by using a standard methodology.

Northern (RNA) blot analysis. Total cellular RNA was purified as described previously by using the standard guanidine thiocyanate-CsCl density centrifugation procedure (24). RNA pellets were redissolved in diethylpyrocarbonate-treated, double-distilled water and precipitated by centrifugation from a 70% ethanol–0.3 M sodium acetate (NaAc, pH 5.2) solution. Pellets were rinsed with 70% ethanol, dried in a vacuum desiccator, and dissolved in 10 mM sodium phosphate buffer (pH 7.0). The RNA concentration was determined by measurement of UV A_{260}. RNA was fractionated by electrophoresis in a 1% agarose gel, transferred by capillary action (39) to Biodyne transfer membranes (Pall Biosupport, East Hills, N.Y.), and immobilized by UV irradiation with a UV cross-linker (Hoefer). Blots were prehybridized for 3 h at 42°C (50% formamide–5× SSC [pH 7.0; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–50 mM $NaPO_4$ [pH 7.0]–250 μg of single-stranded DNA per ml–5× Denhardt's solution–0.1% sodium dodecyl sulfate [SDS]) and hybridized overnight at 42°C with ^{32}P-labeled probes (Multiprime DNA labeling system; Amersham, Arlington Heights, Ill.) in a similar mixture with dextran sulfate added to a final concentration of 5% (39). The hybridized blot was washed twice for 10 min in 2× SSC–0.1% SDS at room temperature, and then three 20-min washes in 0.1× SSC–0.1% SDS at 55°C were performed. Blots were subsequently autoradiographed with preflashed Kodak X-Omat XAR-5 film and an intensifying screen at −70°C for an appropriate period (1 to 3 days). Uniformity of RNA loading on the gels was verified by subsequent hybridization to the appropriate α-tubulin gene probe (23). Experiments were repeated with fresh cells to confirm results.

Reagents and supplies. Dulbecco's modified Eagle's medium was purchased from Whittaker M. A. Bioproducts, Walkersville, Md., and FBS was obtained from HyClone Laboratories, Inc., Logan, Utah. The FBS had total protein of 3.2 g per dl, and it contained 0.06 endotoxin units per ml as determined by the manufacturer. *E. coli* LPS, serotypes O55:B5 and O111:B4, was purchased from Sigma, St. Louis, Mo., and List Biological Laboratories, Inc., Campbell, Calif. PI-PLC was purchased from Boehringer Mannheim, Indianapolis, Ind. Sulfosuccinimidyl-2-(*p*-azidosalicylamido)1,3′-dithiopropionate and chloramine T were from Pierce Chemicals, Rockford, Ill. The $Na^{125}I$ was obtained from ICN, Costa Mesa, Calif. Bio-Rex 70 resin was from Bio-Rad Laboratories, Richmond, Calif., and the YM10 filtration membranes were obtained from Amicon Corp., Danvers, Mass. Chromogenic substrate B2-Ile-Glu-Arg-P-nitroanilide (S-2222) was from Kabi Pharmaceuticals, Franklin, Ohio, and a coagulation factor concentrate (Proplex T) was obtained from Travenol Labs, Inc., Glendale, Calif. Fluorescein isothiocyanate-labeled goat anti-mouse antibody and control antibodies were obtained from Organon Teknika Corp., Malvern, Pa. The two control isotype-matched MAb used in these experiments were mouse IgG1(κ), derived from MOPC 21, a mineral oil-induced plasmacytoma, and mouse IgG2b(κ), derived from MOPC 195, also a mineral oil-induced plasmacytoma. Neither of these have any known hapten or antigen binding activity. The murine anti-human CD14 MAb 3C10 (IgG1) was a generous gift from Samuel D. Wright, Rockefeller University, New York, N.Y. Murine anti-human CD14 MAb 60bd (IgG1, provided as ascites) was a kind gift from Robert F. Todd III, Division of Hematology and Oncology, University of Michigan Medical School, Ann Arbor. MAb 60bd was purified with an ImmunoPure (G) IgG Purification Kit (Pierce) and stored without preservative at −70°C until use. My4, another murine anti-human CD14 monoclonal antibody (IgG2b), was purchased from Coulter Immunology, Hialeah, Fla. MAb 60.3 (anti-CD18) was a kind gift of John Harlan, University of Washington, Seattle. The cDNA probe for human CD14 (34) was generously provided by Brian Seed, Department of Molecular Biology, Massachusetts General Hospital, Boston. The bovine tissue factor cDNA probe (36) was a kind gift from Sadaaki Iwanaga, Kyushu University, Fukuoka, Japan. Salmon sperm DNA and [α-^{32}P]CTP were from DuPont/NEN, Boston, Mass. Other reagents for the Northern blots were purchased from GIBCO/BRL, Grand Island, N.Y.

RESULTS

Immunofluorescence flow cytometric analysis. Anti-human CD14 MAb were tested for cross-reactivity with bAM, and three MAb (3C10, 60bd, and My4) were found to bind on the basis of fluorescence flow cytometric analysis (data not shown). Membrane-associated CD14 is a glycosyl phosphatidylinositol-anchored receptor on human cells (16, 34), and we therefore tested the effect of PI-PLC pretreatment of bAM prior to antibody labeling. PI-PLC pretreatment reduced the relative fluorescence intensity of antibody-labeled cells, indicating that binding of 3C10 MAb to bAM was reduced in a dose-dependent manner (Fig. 1A). Whereas pretreatment of macrophages with PI-PLC decreased binding of the two anti-CD14 MAbs tested (3C10 and MY4), PI-PLC treatment did not decrease binding of MAb 60.3, which recognizes the CD18 leukocyte integrin (Fig. 1B). CD18 is not a phospholipid-anchored re-

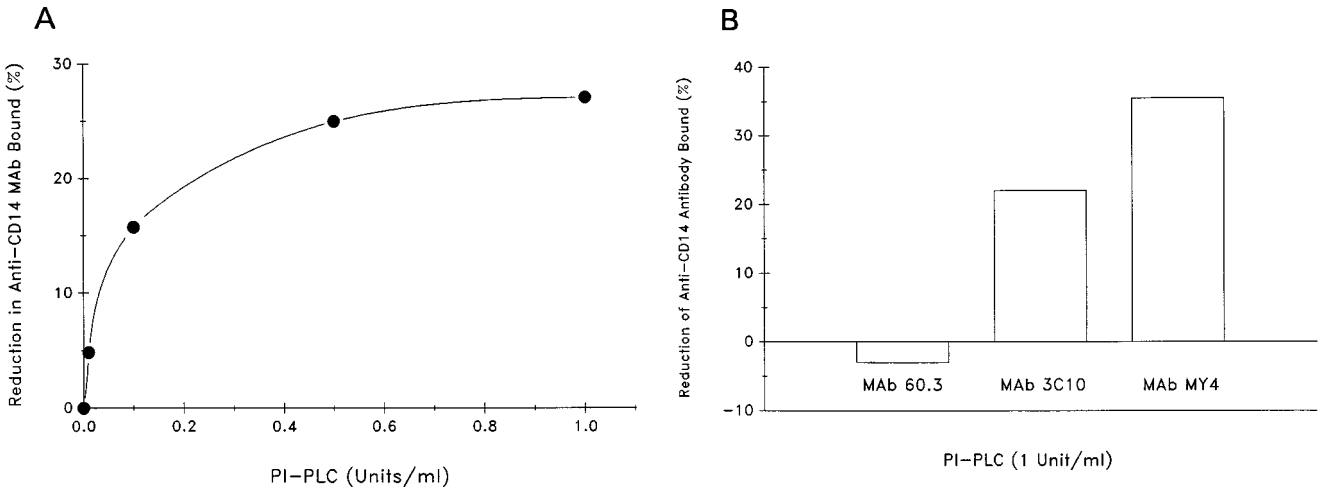

FIG. 1. Binding of anti-CD14 MAb to bAM is decreased by PI-PLC pretreatment. (A) Macrophages were preincubated with several concentrations of PI-PLC (0.1 to 1.0 U/ml), and then indirect immunofluorescence flow cytometry using MAb 3C10 was performed. (B) Macrophages were exposed to a single concentration of PI-PLC (1.0 U/ml) and then similarly labeled with anti-CD14 MAb (3C10 or MY4; 10 μg/ml) or anti-CD18 MAb (60.3; 10 μg/ml) and analyzed by indirect immunofluorescence flow cytometry. Results are expressed as percents reduction of antibody binding. Results from one of three similar experiments are shown.

ceptor. Experiments were repeated three times; results from one experiment are shown in Fig. 1A and B.

Anti-CD14 MAb decreases binding of ^{125}I-labeled LPS to macrophages. Previous reports indicate that binding of LPS to human macrophages is dependent upon mCD14 and soluble serum factors such as LPS-binding protein. In order to investigate the requirements for binding of LPS to bAM, binding of ^{125}I-labeled LPS to bAM was quantitated in the presence of serum and anti-CD14 MAb. Association of LPS with bAM was dependent on the concentration of LPS (Fig. 2A). At all concentrations of LPS, the amount of cell-bound LPS was markedly increased by the presence of bovine serum (Fig. 2A). We employed one of the anti-CD14 MAb that had already been shown to bind to bAM in these assays (MAb 60bd; 20 μg/ml) and found that the anti-CD14 antibody decreased association of LPS with macrophages (Fig. 2B). Anti-CD14 MAb 60bd diminished total binding of ^{125}I-labeled LPS to bAM by 43% in the presence of 0.5% FBS and by 24% with 1.0% FBS, and isotype-matched MAb of irrelevant specificity did not interfere with LPS binding. Results shown in Fig. 2A and B are means for duplicate samples from one of two similar experiments.

Northern blot analysis of bovine macrophage CD14 mRNA. bAM were stimulated with LPS (1 ng/ml) with 0.2% FBS for periods of time up to 24 h, and a CD14 cDNA probe based on the human sequence was hybridized with RNA purified from the bAM. RNA purified from human monocytes was included as a control in the experiments. The conditions of stringency for posthybridization washing were 0.1× SSC with 0.1% SDS buffer for three washes at a water bath temperature of 55°C. As shown in Fig. 3, a band of the expected size (1.5 kb) was present in human monocyte RNA (left) and in RNA from bAM (right). Unexpectedly, an additional 3.1-kb band appeared to be associated with the bovine cells. The level of CD14 mRNA expression appeared greatest in bAM at 24 h after stimulation with LPS.

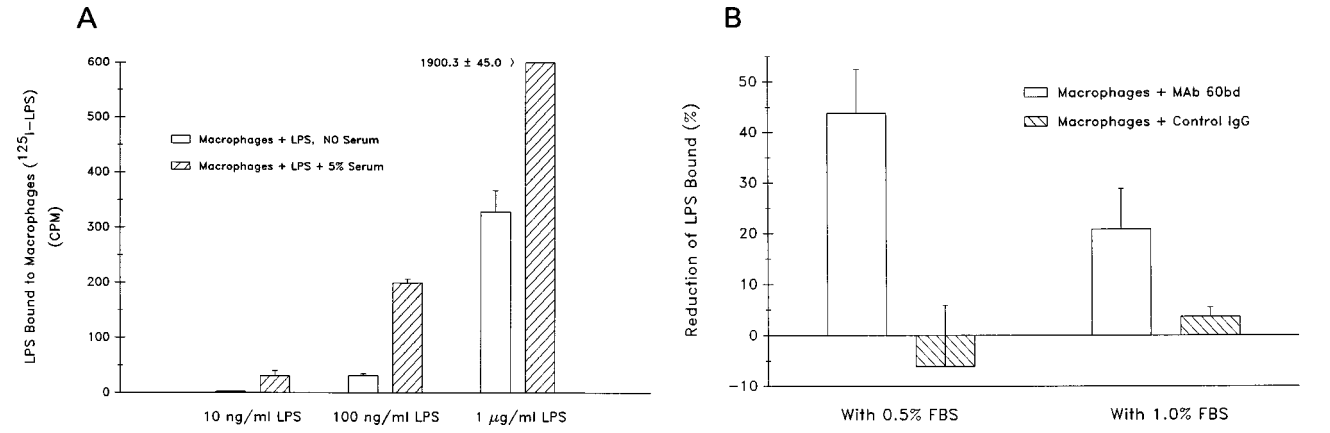

FIG. 2. Binding of ^{125}I-labeled LPS to macrophages. (A) Association of several concentrations of ^{125}I-labeled LPS with bAM in the presence and absence of normal pooled bovine serum (5%) was quantitated in order to determine whether soluble serum components influence binding of LPS to cells. Results are expressed as LPS-associated gamma counts (cpm) from macrophages. (B) The effects of anti-CD14 MAb (60bd; 20 μg/ml) and an isotype-matched MAb of irrelevant specificity on binding of ^{125}I-LPS (100 ng/ml) to macrophages in the presence of soluble serum components are shown. Results are expressed as percents reduction of LPS bound to antibody-treated macrophages, as determined from LPS-associated gamma counts. Results shown in panels A and B are means ± standard deviations for duplicate samples from one of two similar experiments.

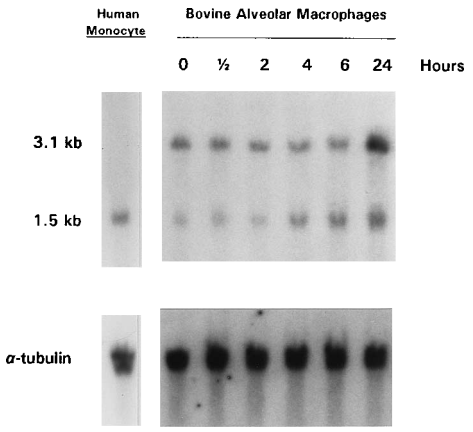

FIG. 3. Northern blot analysis of bovine CD14 mRNA from bAM. A band of the expected size (1.5 kb) was present in RNA from human monocytes (left lane), while RNA from bAM showed the expected 1.5-kb band and an additional 3.1-kb band. bAM were stimulated for up to 24 h with LPS (1 ng/ml) and FBS (0.2%) before RNA was purified. Results were similar for two blots derived from separate isolates of macrophages.

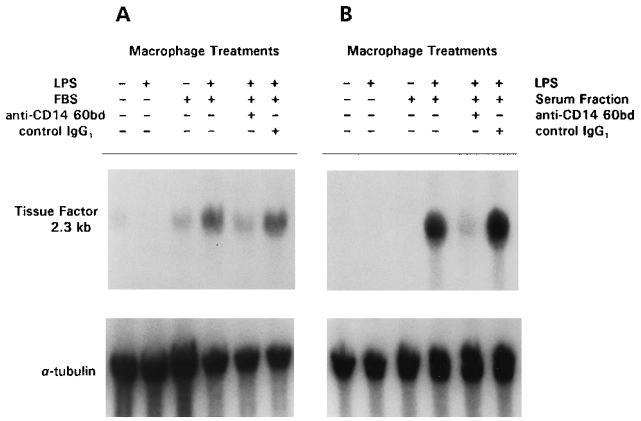

FIG. 5. Regulation of tissue factor mRNA expression by macrophages. Experiments were conducted by using macrophage treatment variables including the presence or absence of LPS (1 ng/ml), FBS (0.2%) (A), bovine serum-derived chromatography fraction (0.5 μg/ml of protein) (B), anti-CD14 MAb 60bd (10 μg/ml), and an isotype-matched MAb of irrelevant specificity. +, treatment was used; −, treatment was not used. After 5 h of incubation of cells, RNA was purified and subsequently probed with bovine tissue factor cDNA. Experiments were repeated twice to confirm results.

Expression of tissue factor on macrophages was inhibited by anti-CD14 MAb. The chromogenic assay based on activation of factor X was used to assess tissue factor expression as a measure of macrophage activation. The results that we obtained by utilizing the chromatographically derived bovine serum fraction were similar to those previously obtained in our laboratory (50). In a manner similar to that of the previous results, current experiments utilizing FBS indicated that components in FBS markedly enhanced the stimulatory effect of LPS (1 ng/ml) but that LPS alone had little stimulatory effect (data not shown). Stimulation of macrophages was inhibitable by treatment of bAM with anti-CD14 MAb (60bd; 20 μg/ml) but not by treatment with an isotype-matched MAb of irrelevant specificity (20 μg/ml). A similar inhibitory effect was also observed when another anti-CD14 MAb, MY4, was employed.

Regulation of tissue factor mRNA expression by macrophages. For Fig. 4, alveolar macrophages were stimulated for various periods with LPS (1 ng/ml) plus either FBS (0.2%; 64 μg/ml of protein) (A) or a bovine serum-derived chromatography fraction (0.5 μg/ml of protein) (B), and RNA was purified. Northern blot analysis revealed that tissue factor mRNA expression was strongest from 4 to 8 h poststimulation and that it diminished after 24 h. Similar experiments were conducted by using a single time point of 5 h poststimulation and treatment variables including the presence or absence of LPS (1 ng/ml), FBS (0.2%), bovine serum-derived chromatography fraction (0.5 μg/ml), anti-CD14 MAb 60bd (10 μg/ml), and an isotype-matched MAb of irrelevant specificity (Fig. 5). bAM alone, bAM in 0.2% FBS alone, and bAM treated with 1 ng of LPS per ml in the absence of FBS showed an absence or the presence of low levels of tissue factor mRNA transcripts (A). When bAM were treated with 1 ng of LPS per ml in the presence of 0.2% FBS, the tissue factor mRNA level was remarkably increased, with the expected band appearing at 2.3 kb. Tissue factor mRNA levels were lower when cells were treated with anti-CD14 MAb 60bd (10 μg/ml) but not when they were treated with the isotype-matched MAb of irrelevant specificity. Similar results were obtained when the bovine serum chromatography fraction was employed (B), except that the serum protein fraction alone induced no signal, whereas FBS alone (A) had a slight stimulatory effect. These results indicated that a CD14-like receptor on bAM participated in LPS-plus serum protein-induced tissue factor mRNA expression.

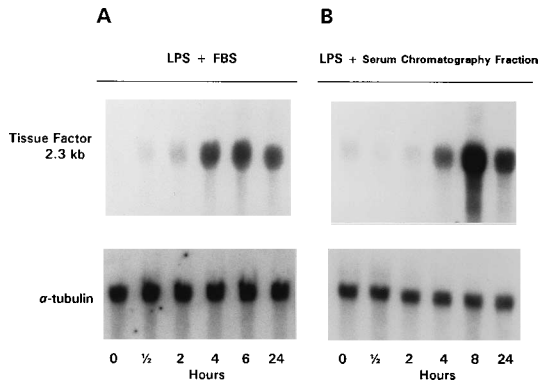

FIG. 4. Kinetics of expression of tissue factor mRNA by macrophages. Alveolar macrophages were stimulated for periods of up to 24 h with LPS (1 ng/ml) plus either FBS (0.2%; 64 μg/ml of protein) (A) or a bovine serum-derived chromatography fraction (0.5 μg/ml of protein) (B). Purified RNA was then probed by using bovine tissue factor cDNA. Experiments were repeated twice to confirm results.

DISCUSSION

Bacterial LPS in the blood of mammals may bind to several different serum components, but binding to LBP and soluble CD14 is notable because of its high avidity (14, 32, 44). Binding of LPS to LBP accelerates subsequent delivery of LPS to soluble CD14 (14), and LBP-LPS complexes also interact with mCD14 on monocytes, macrophages, and neutrophils (18, 32, 42, 45). This serves to enhance the sensitivity of responsive cells to the presence of LPS (10, 12, 17, 25, 32), but overresponsiveness may also precipitate immune system-mediated septic shock (11, 28–30). We have focused our current work on alveolar macrophages because these cells respond to bacterial LPS in combination with soluble proteins of either pulmonary or vascular origin, and investigators have demonstrated that

LBP and soluble CD14 are present in some cases of acute pulmonary inflammation (25, 26).

Investigators utilizing human monocytes (16) or CD14-transfected cell lines (34) have demonstrated that treatment of cells with PI-PLC reduces binding of anti-CD14 MAb. This is attributable to the fact that the glycosyl phosphatidylinositol anchor of mCD14 is susceptible to cleavage by specific phospholipase C, and we have similarly shown that binding of anti-human CD14 MAb to bAM is reduced by pretreatment of bAM with PI-PLC. This suggests that the antigen on bovine cells recognized by the antibodies has a phospholipid anchor, as is the case with mCD14. mCD14 is known to serve as a receptor for LPS on human and rabbit macrophages (25, 32). Incomplete removal of the putative mCD14 on bovine macrophages in our experiments may have been due to cleaved CD14 staying on the cells in a noncovalently attached form or to use of an inadequate concentration of PI-PLC in the experiments. We have also found that radiolabeled LPS binds to bAM and that the presence of serum enhances binding of LPS to the cells. In addition, anti-CD14 MAb decreased binding of LPS to bAM in the presence of serum. Our findings with bovine cells are consistent with the mechanism of interaction of LPS with monocytes and macrophages of other species, which dictates that LPS binds to LBP in serum and enhances binding to mCD14 on the surfaces of cells (42, 44). Our findings with lung-derived bovine macrophages are also in concert with those of other investigators who found that rabbit and human alveolar macrophages respond to LPS and LBP in an mCD14-dependent manner (25). The inability of anti-CD14 MAb to completely block binding of radiolabeled LPS to macrophages in our experiments may have been due to the presence of other (non-CD14) LPS receptors or LPS-receptive proteins on the macrophage surface.

Our studies indicate that mRNA encoding the mCD14 receptor is present in bAM, which is consistent with the findings we obtained when we used anti-CD14 MAb which bind to these cells. The bovine mRNA appeared as a 1.5-kb band, which was similar in size to that observed with the human monocyte CD14 mRNA used as a control and to that reported for human CD14 in the literature (34). The additional 3.1-kb band on the blots from bovine cells was unexpected, and its significance is unknown. We also found that the mRNA transcript level for CD14 in bovine macrophages was increased after stimulation with LPS for 24 h. Levels of mCD14 receptor transcripts and surface receptor expression in the human monocytic Mono Mac 6 cell line have been reported to be increased by stimulation with LPS or by prostaglandin E_2, but transiently decreased (51) or increased (38) by phorbol ester. Low concentrations of LPS (0.1 to 1.0 ng/ml) increased mCD14 receptor expression on human monocytes, but expression, as measured by flow cytometry, was variably decreased with high LPS concentrations (100 ng/ml) (20). Increased levels of mRNA transcripts for mCD14 are related to both increased surface expression of mCD14 on cells and increased shedding of CD14 into the extracellular fluid (51).

Our data also indicated that activation of bAM by LPS was enhanced by the presence of bovine serum or soluble bovine serum factors and that activation was inhibitable by anti-CD14 MAb. Tissue factor mRNA levels in bAM were elevated by 2 h of stimulation with LPS, peaked after 6 to 8 h, and decreased slightly by 24 h. Results of Northern blot analysis of tissue factor mRNA levels from bAM were consistent with the findings obtained from cell assays measuring tissue factor expression, and they indicated that LPS-induced tissue factor synthesis is dependent upon mCD14. Tissue factor is a transmembrane protein, and it functions as a major cellular initiator of the clotting cascade (1). Increased expression of tissue factor is related to the pathogenesis of gram-negative bacterial diseases, and it is a marker of LPS-mediated macrophage activation (7, 37). Our finding that LPS-mediated activation of bAM is dependent on CD14 is comparable to previous findings of CD14-dependent stimulation of human and rabbit macrophages by LPS and LBP (10, 25, 49).

In summary, our findings indicate the existence of mCD14 on bAM. Results indicate that bAM are dependent on an mCD14-like receptor and soluble serum factors for LPS-mediated cellular activation and tissue factor expression, elucidating likely pathways of pulmonary inflammation in bovine pneumonia due to gram-negative bacteria. The data also indicate that the function of the proposed bovine mCD14 receptor is comparable to that reported for human mCD14, suggesting conservation of mechanisms of macrophage responsiveness to bacterial LPS.

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