INFECTION AND IMMUNITY, Feb. 1995, p. 381–388 Vol. 63, No. 2
0019-9567/95/$04.00+0

Induction of Inflammatory Cytokines in Bovine Alveolar Macrophages following Stimulation with *Pasteurella haemolytica* Lipopolysaccharide

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Received 27 July 1994/Returned for modification 31 August 1994/Accepted 1 November 1994

Bovine tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) cDNAs were generated by reverse transcription and then by PCR amplification from lipopolysaccharide (LPS)-stimulated alveolar macrophage RNA. The amplified cDNAs were cloned into pPow and expressed in *Escherichia coli* DH5α. The expressed proteins were confirmed as TNF-α and IL-1β by Western blot (immunoblot) analysis and bioassays. We then used the cloned genes as probes in Northern (RNA) blots and investigated the kinetics of TNF-α and IL-1β mRNA expression in bovine alveolar macrophages stimulated with purified LPS from *Pasteurella haemolytica* 12296. The effect of LPS on TNF-α and IL-1β gene expression was dose dependent, and induction was observed at a concentration of 0.01 μg/ml. Both TNF-α and IL-1β mRNA expression were detectable within 0.5 h after stimulation with 1 μg of LPS per ml, peaked at 1 to 2 h, steadily declined up to 16 h, and were undetectable by 24 h. Secreted TNF-α measured by bioassay peaked at 4 h and accumulated at a lesser concentration in conditioned medium throughout the 24 h. By contrast, secreted IL-1β was induced at 8 h and reached a maximal concentration at 24 h after stimulation. The ability of LPS to induce TNF-α and IL-1β gene expression and secretion of bioactive proteins were suppressed by polymyxin B. Our findings support a role for LPS from *P. haemolytica* in the induction of inflammatory cytokines in bovine pneumonic pasteurellosis.

Shipping fever or bovine pneumonic pasteurellosis is the disease that causes the greatest economic losses in the North American cattle industry (3, 45). It is believed to be caused by interaction of stressful management practices, environmental factors, and a variety of microorganisms. The primary bacterial agent that is responsible for the clinical disease and pathophysiological events characterized by acute lobar fibrinonecrotizing pneumonia is *Pasteurella haemolytica* biotype A serotype 1 (A1) (12, 29). Morphological studies (21, 31, 38, 42, 43) suggest that early in infection, an influx of neutrophils into the alveoli occurs and then extensive edema fluid containing fibrin in the alveoli, on the pleural surface, and in the interlobular septa accumulates. Other lesions which appear later include hemorrhage, vascular thrombosis, coagulative parenchymal necrosis, and abscess formation. These lung lesions have been documented in both naturally occurring (29) and *P. haemolytica*-induced experimental pneumonic pasteurellosis (4).

P. haemolytica produces several potential virulence factors, including lipopolysaccharide (LPS) with endotoxic properties, an exotoxic leukotoxin (Lkt), neuraminidase, capsular polysaccharide, and a neutral protease that may be involved in the initial injury in the lung (10). Of these factors, LPS and Lkt have received the most attention. Whiteley et al. (42, 43) and others (11, 32) have shown that intrabronchial or intratracheal inoculation of calves with purified LPS from *P. haemolytica* A1 causes neutrophil influx, fibrin exudation, and edema in the alveolar spaces and platelet and neutrophil aggregation in capillaries, which lead to pathophysiologic derangements in the lung. By using immunohistochemical techniques on pneumonic lung sections, Whiteley et al. showed previously (41) the in situ presence of LPS in neutrophils in the alveolus and interstitial and capillary lumina, in intravascular, interstitial, and alveolar macrophages (AMs), in endothelial cells, and on epithelial cell surfaces. Morphological studies (42, 43) also showed that AMs exposed to LPS showed changes characteristic of cell activation. These and other results suggest that AMs play a central role in orchestrating the inflammatory cascade in the lung and lead to tissue injury in bovine pneumonic pasteurellosis.

Studies using animal models of sepsis-related human adult respiratory distress syndrome have helped define the contribution of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), and IL-8 in the pathogenesis of acute lung injury (6, 17, 18, 27, 33, 37). These cytokines act as chemotactic factors and induce neutrophil influx into an inflammatory site because of their ability to directly induce chemotaxins and to indirectly induce the production of lipid chemotaxins (7). Furthermore, they induce expression of adhesion molecules on endothelial cells and neutrophils (6, 7) and augment endothelial cell injury induced by activated neutrophils (30). There is compelling evidence which suggests that IL-8 is a more powerful neutrophil chemotactic cytokine than the other cytokines and that it promotes the recruitment and activation of neutrophils into the alveolar spaces and plays a major role in the pathophysiology of human adult respiratory distress syndrome (6, 19). These findings are especially relevant since the pathophysiological alterations associated with lung injury in bovine pneumonic pasteurellosis resembles human adult respiratory distress syndrome lung injury (39).

We are interested in studying the role of AM-derived inflammatory cytokines in the pathogenesis of lung injury in bovine pneumonic pasteurellosis. In this context, the cytokine-

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TABLE 1. Oligonucleotides of upstream (5′) primers and downstream (3′) primers of target genes

Target gene	Oligonucleotide sequence	Size of PCR product (bp)
TNF-α	5′ primer-CGGATCCA AGG TCC TCT TCT CAA GCC TC *Bam*HI	488
	3′ primer-TGAATTC TA CAG GGC GAT GAT CCC AAA G *Eco*RI	
IL-1β	5′ primer-CGGATCC A GCA CCC GTT CAG TCA ATA AAG *Bam*HI	474
	3′ primer-CGAATTC TA GGG AGA GAG GGT TTC CAT TC *Eco*RI	

inducing capabilities of various virulence factors of *P. haemolytica* A1 have not been examined in vitro. As a first step, the purpose of the present study is to characterize the expression of TNF-α and IL-1β from bovine AMs stimulated by the LPS from *P. haemolytica* A1.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *Escherichia coli* DH5α and pBluescript KS(+) were obtained from D. W. Anderson, Department of Oral Sciences, University of Minnesota. The pPow expression vector was obtained from M. P. Murtaugh, Department of Veterinary PathoBiology, University of Minnesota. *E. coli* DH5α transformed with recombinant pBluescript KS(+) was grown in Luria-Bertani medium, which contained 10 g of Bacto Tryptone, 5 g of yeast extract, and 10 g of sodium chloride in 1,000 ml of distilled water, while *E. coli* transformed with recombinant pPow was grown in superbroth medium, which contained per liter 20 g of Bacto Tryptone, 10 g of yeast extract, 5 g of sodium chloride, 2.5 g of K_2HPO_4, 1 g of $MgSO_4 \cdot 7H_2O$, 1 ml of 0.1% biotin, 1 ml of 1% thiamine, 3 ml of trace element solution (1.6 g of $FeCl_3$, 0.2 g of $ZnCl_2 \cdot 4H_2O$, 0.2 g of $CoCl_2 \cdot 6H_2O$, 0.2 g of $Na_2MoO_4 \cdot 2H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$, 0.1 g of $CuCl_2$, 0.05 g of H_3BO_3, 10 ml of 12 N HCl, and distilled water up to 100 ml), and 100 μg of ampicillin in 1,000 ml of distilled water.

Recovery and isolation of AMs. Healthy 6- to 8-week-old calves were purchased from the Department of Animal Science, University of Minnesota. Calves were preanesthetized with an intravenous injection of 0.5 ml of xylazine hydrochloride (Miles Inc., Shawnee Mission, Kans.) and euthanatized with an overdose of barbiturate. AMs were collected by lung lavage with sterile, endotoxin-free phosphate-buffered saline (PBS; pH 7.4). Approximately 2×10^7 cells were plated onto 6-cm tissue culture petri dishes and cultured overnight at 37°C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle medium (DMEM; Sigma Chemical Co., St. Louis, Mo.) supplemented with 2% fetal bovine serum (Irvine Scientific, Santa Ana, Calif.), 1 mM L-glutamine, 0.1 mM nonessential amino acids, 14 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 2.2 g of $NaHCO_3$ per liter, 100 U of penicillin per ml, and 0.1 mg of streptomycin per ml. Nonadherent cells were removed by washing twice with PBS, and adherent AMs were incubated with fresh medium as described for individual experiments. Adherent populations were >95% macrophages and >98% viable as determined by nonspecific esterase staining and trypan blue dye exclusion, respectively. Inducers or treatments were added directly to the existing media the following morning to avoid nonspecific stimulation of quiescent cells.

Stimulation of AMs. To stimulate the AMs, cells were cultured in the presence of purified LPS from *P. haemolytica* 12296. The LPS was isolated by the hot phenol-water extraction technique (40). It was found to be pure and did not contain any polypeptide bands when examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then by silver staining (36). Concentration of endotoxin present in the LPS as determined by a *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, Md.) revealed that 1 μg of LPS per ml was equivalent approximately to 200 endotoxin units. Concentrations of LPS and exposure times varied as described for individual experiments. In the experiments with polymyxin B (Sigma), AMs were preincubated with 1 ml of DMEM containing 1 or 10 μg of polymyxin B per ml for 30 min, 1 μg of LPS per ml was added, and the mixture was incubated at 37°C for 4 h. In some experiments, polymyxin B was mixed with LPS and the mixture was added to AMs. Polymyxin B appeared to be toxic (by trypan blue exclusion test) to bovine AMs at a concentration of 50 μg/ml or higher but not at 1 to 25 μg/ml. Thereafter, the culture medium was collected and centrifuged for 10 min at 1,000 × *g*. The pelleted cells were discarded, while the supernatants referred to as AM-conditioned media were aliquoted and frozen at −70°C until further testing for TNF-α and IL-1 bioactivity.

Cytokines, antibodies, and reagents. Purified recombinant bovine TNF-α (rbTNF-α), polyclonal antibodies against rbTNF-α produced in rabbit (anti-bovine TNF-α), and preimmune rabbit serum were generously provided by T. H. Elsasser, U.S. Department of Agriculture, Beltsville, Md. Polyclonal antibodies against recombinant bovine IL-1β (rbIL-1β) were raised by immunizing specific-pathogen-free chickens in our laboratory. Five- to 6-week-old chickens were injected intramuscularly with 1 ml of rbIL-1β (100 μg/ml; American Cynamide, Princeton, N.J.) emulsified with Freund's complete adjuvant at a 1:1 ratio. Two weeks after the first injection, the chickens were given a second intramuscular injection of 1 ml of rbIL-1β (100 μg/ml) emulsified with incomplete Freund's adjuvant at a 1:1 ratio. Two weeks after the booster, the chickens were bled by exsanguination, and serum (anti-bovine IL-1β) was collected and stored at −70°C until used. Specificity of anti-bovine TNF-α and anti-bovine IL-1β were confirmed by Western blot (immunoblot) analysis (35). Recombinant human IL-1 receptor antagonist (IL-1ra) was purchased from R&D Systems (Minneapolis, Minn.).

RNA extraction. Total RNA was extracted from AMs cultured in the presence or absence of LPS from *P. haemolytica* 12296, with or without polymyxin B, by the acid guanidinium thiocyanate method as described by Chomczynski and Sacchi (8). Concentrations of LPS and polymyxin B and exposure times varied as described for individual experiments. All solutions used for RNA extraction were treated with 0.05% diethylpyrocarbonate and then autoclaved to remove the diethylpyrocarbonate. Glassware was baked overnight at 200°C. Total RNA was quantified by measuring the A_{260} with a spectrophotometer (model DU-64; Beckman Instruments, Inc., Fullerton, Calif.) and checked by agarose gel electrophoresis. The RNA samples were stored in 2.5 volumes of ethanol at −70°C until used.

Cloning of bovine TNF-α and IL-1β cDNA. (i) Synthesis of cDNA. Contaminating DNA was removed from total RNA with RNase-free DNase I (Gibco BRL, Grand Island, N.Y.) by incubation at 37°C for 15 min. One microliter of oligo(dT) primer (dT_{12-17}; Gibco BRL) was added to 5 μg of DNase I-treated RNA dissolved in 13 μl of diethylpyrocarbonate-treated water. The mixture was heated at 70°C for 10 min and chilled on ice. Synthesis of cDNA was carried out in 20 μl of solution containing 14 μl of RNA and oligo(dT) primer mixture, 2 μl of 10× synthesis buffer (Gibco BRL), 2 μl of 0.1 M dithiothreitol, 1 μl of a 10 mM deoxynucleoside triphosphate (dNTP) mixture, and 1 μl of murine leukemia virus reverse transcriptase (Gibco BRL) at 42°C for 1 h. RNA was removed by treatment with RNase H (Gibco BRL) at 37°C for 20 min. The synthesized single-stranded cDNA sample was stored at −20°C until used.

(ii) PCR and cloning of amplified products. PCR was performed for 30 cycles (93°C for 1 min, 52°C for 30 s, and 72°C for 30 s) with the synthesized single-strand cDNA and primers shown in Table 1 in 25 μl of a solution containing 2.5 μl of 10× amplification buffer (Perkin-Elmer Cetus, Norwalk, Conn.), 0.8 μl of 2.5 mM each dNTP mix solution, 1 μl of upstream primer (100 ng/μl), 1 μl of downstream primer (100 ng/μl), and 0.2 μl of *Taq* DNA polymerase (Perkin-Elmer Cetus). Primers for PCR amplifying bovine TNF-α and IL-1β were synthesized by use of the information obtained from GenBank on coding sequences of mature bovine TNF-α and IL-1β proteins. The primers contained *Eco*RI and *Bam*HI restriction sites and were synthesized on an Applied Biosystems (Foster City, Calif.) model 391 DNA synthesizer. The products of amplification were digested with *Eco*RI and *Bam*HI and separated on a 2% agarose gel. The fragments were purified with GenecleanII (Bio 101, Inc., LaJolla, Calif.), ligated into *Eco*RI- and *Bam*HI-digested pBluescript KS(+), and transformed into *E. coli* DH5α. The cloned fragments were analyzed by restriction enzyme digestion with *Acc*I, *Bgl*I, *Hae*III, *Hin*cII, and *Pvu*II for TNF-α and *Acc*I, *Hae*III, *Pst*I, and *Pvu*II for IL-1β. Inserts confirmed by restriction enzyme analysis that they were indeed bovine TNF-α and IL-1β genes (9, 22) were then subcloned into the pPow expression vector.

(iii) Expression of rbTNF-α and rbIL-1β. *E. coli* DH5α harboring native pPow vector or recombinant pPow carrying TNF-α or IL-1β cDNA inserts was grown in superbroth to express the proteins. The bacteria grown in superbroth overnight at 30°C were diluted into fresh medium at a 1:10 dilution and incubated with shaking at 30°C for an additional 4 h. Heat induction to express the proteins was carried out by exposure of the bacteria to 42°C for 15 min and then incubation at 37°C for 4 h.

(iv) Analysis of the expressed rbTNF-α and rbIL-1β. The intracellular and extracellular expression of rbTNF-α and rbIL-1β was analyzed by SDS-PAGE

and Western blotting. To determine whether the expressed recombinant cytokines were extracellular proteins, 900 μl of culture supernatant was precipitated with 100 μl of 50% trichloroacetic acid while keeping the solution on ice for 1 h. The proteins were collected by centrifugation, washed with cold acetone, and resuspended in 100 μl of SDS-PAGE sample buffer. To determine whether the expressed cytokines were intracellular, a 1-ml culture of *E. coli* DH5α transformed with pPow or pPow carrying the TNF-α or IL-1β cDNA insert was harvested by centrifugation, washed with PBS, resuspended in 150 μl of SDS-PAGE sample buffer, and incubated in boiling water for 5 min. The samples were then subjected to SDS-PAGE using a 12% acrylamide gel (20). After electrophoresis, the gels were either stained with Coomassie brilliant blue R-250 or transferred electrophoretically onto nitrocellulose membranes and processed for Western blotting (35). The membranes were blocked with TBS buffer (50 mM Tris, 150 mM NaCl [pH 7.5]) supplemented with 5% nonfat dried skim milk and probed with anti-bovine TNF-α or anti-bovine IL-1β diluted in the same buffer at 1:1,000 and 1:100, respectively. The IL-1β-specific band was detected by incubation with goat anti-chicken immunoglobulin G-horseradish peroxidase (Cappel, Durham, N.C.) and then by incubation with a substrate solution containing 0.06% (wt/vol) 4-chloro-1-naphthol and 0.06% (vol/vol) hydrogen peroxide. A TNF-α-specific band was detected by incubation with goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Southern Biotechnology Associates, Inc., Birmingham, Ala.) and then by detection with nitroblue tetrazolium chloride and χ-phosphate solution. Biological activities of the cytokines expressed in *E. coli* DH5α were quantified with WEHI 164 clone 13 cells for TNF-α and the D10 (N4) M murine T-cell line (subline of D10.G.4.1) as target cells for IL-1β as described below. Filter-sterilized culture supernatants from *E. coli* harboring native pPow vector or pPow carrying the TNF-α or IL-1β cDNA insert were used for confirmation of the biological activities after expression of the genes by heat induction. The protein concentration in the culture supernatants was determined with the DC protein assay kit (Bio-Rad Laboratories, Hercules, Calif.) as described in the instruction manual. The culture supernatants were diluted in superbroth to the same protein concentration and then subjected to the bioassays.

Preparation of Northern (RNA) blot probes. Recombinant pBluescript KS(+), which carried either cloned bovine TNF-α or IL-1β cDNA inserts, was digested with *Eco*RI and *Bam*HI and electrophoresed on a 1.5% agarose gel, and the inserts were purified with GenecleanII. The purified inserts were random primed with [α-^{32}P]dCTP (Amersham, Arlington Heights, Ill.) with the RadPrime Labeling System (Gibco BRL) by following the established protocols in the instruction manual. After labeling, unincorporated [α-^{32}P]dCTP was removed by passing the mixture over a Sephadex G-25 (Sigma) spun column at 5,000 rpm for 5 min in a microcentrifuge. One microliter of flowthrough was counted in a scintillation counter, and the probes were adjusted to achieve 2×10^6 cpm/ml.

Kinetics of TNF-α and IL-1β mRNA expression. Sample RNA extracts (5 μg) were electrophoresed on a 1% agarose–2.2 M formaldehyde gel and transferred to a Hybond TM$^+$ nylon membrane (Amersham) in 20× SSC (1× SSC is 150 mM NaCl and 15 mM sodium citrate [pH 7.0]). Prehybridization and hybridization were performed with Rapid hyb buffer (Amersham) as described in the instruction protocol. Briefly, prehybridization was carried out at 65°C for 30 min. Hybridization was performed with ^{32}P-labeled bovine TNF-α and IL-1β cDNA probes (specific activity, $\geq 2 \times 10^8$ cpm/μg of DNA) at 2×10^6 cpm/ml for 2 h. The membranes were washed with 2× SSC–0.1% SDS at room temperature for 20 min and twice with 0.2× SSC–0.1% SDS at 65°C for 15 min before autoradiographic exposure. The membranes were autoradiographed by exposure to X-ray film (Kodak) with Lightning-Plus intensifier screens (Kodak) at −80°C for 3 to 8 h. Northern blot bands were quantified by densitometric scanning with a Shimadzu CS-9000 scanning densitometer (Shimadzu Corp., Kyoto, Japan) and normalized to the mRNA level of the constitutively expressed enzyme glyceraldehyde phosphate dehydrogenase in the same blot.

Bioassays. (i) TNF-α bioassay. TNF-α was measured by the bioassay using WEHI 164 clone 13 cells described by Adams and Czuprynski (1) with few modifications. Conditioned media to be tested for bioassay were diluted 2- or 10-fold in DMEM containing 2% fetal bovine serum, and 100 μl from each dilution was added to triplicate wells containing WEHI cells grown to confluency. Recombinant human TNF-α (1, 5, 10, 50, 100, and 250 pg/ml; R&D Systems) and DMEM containing 2% fetal bovine serum were included as positive and negative controls, respectively. The 96-well plates were then incubated at 37°C in a humidified atmosphere containing 5% CO_2 overnight. Twenty microliters of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) dissolved in PBS at 5 mg/ml was added to each well. The plates were allowed to incubate for an additional 3 to 4 h at 37°C and then centrifuged at 1,000 × *g* for 10 min to pellet formazan crystals produced by viable cells. The supernatants were discarded, and formazan crystals were solubilized in 100 μl of lysing solution (50% dimethylformamide, 20% SDS [pH 4.7]) per well. After 1 h, the optical density was measured at 570 nm with a microplate enzyme-linked immunosorbent assay reader (Molecular Device Corp., Menlo Park, Calif.). Concentrations of TNF-α were expressed in picograms per milliliter, calculated by extrapolation based on a standard curve established with recombinant human TNF-α.

(ii) IL-1 bioassay. IL-1 was measured by use of the D10 (N4) M murine T-cell line (subline of D10.G.4.1) as reported previously (44). Test samples were added at various dilutions in triplicate to 96-well plates containing 10^4 cells per well. Recombinant human IL-1β (33, 11, 3.6, 1.2, 0.4, and 0.13 pg/ml; R&D Systems) and RPMI 1640 medium were used as positive and negative controls, respectively. Plates were incubated for 3 days in a humidified incubator at 37°C in the presence of 5% CO_2. After 3 days, MTT dye solution (5 mg/ml) was added to each well at a volume of 25 μl, and the wells were incubated for an additional 4 h. MTT formazan crystals were solubilized by the addition of 50 μl of lysing solution and then incubated overnight at 37°C in the presence of 5% CO_2, and the optical density was measured at 570 nm. The proliferative responses were transformed into picograms per milliliter by extrapolation based on a standard curve established with recombinant human IL-1β.

Statistics. Results from bioassays were expressed as the means ± standard deviations. Analysis of significance was performed by Student's *t* test.

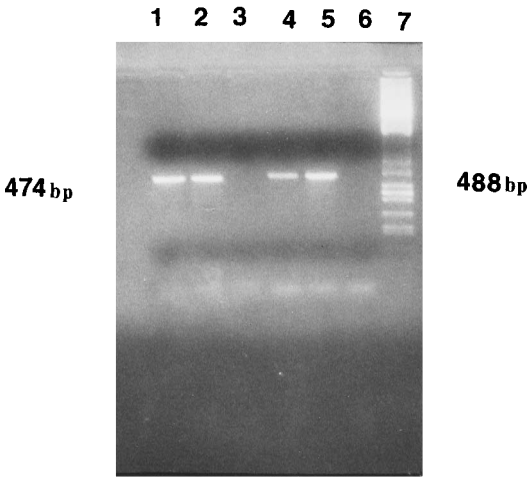

FIG. 1. Agarose gel electrophoresis (using 2% agarose) and ethidium bromide staining were used to analyze products from PCR of an LPS-stimulated bovine AM cDNA library with sets of primers shown in Table 1. Lanes: 1 and 2, 474-bp amplified gene fragment encoding mature bovine IL-1β; 4 and 5, 484-bp fragment encoding mature TNF-α; 3 and 6, buffer controls for IL-1β and TNF-α, respectively, containing all reaction components except cDNA; 7, 1-kb ladder DNA size marker standard.

RESULTS

Cloning of bovine TNF-α and IL-1β cDNA. By using the primers shown in Table 1, bovine TNF-α and IL-1β genes encoding mature proteins were amplified as 488- and 474-bp fragments, respectively (Fig. 1). The amplified fragments were purified and ligated into pBluescript KS(+) and transformed into *E. coli* DH5α. After verification of the restriction enzyme map of the cloned fragments that they were bovine TNF-α and IL-1β (9, 22), 1 clone encoding TNF-α and 31 clones encoding IL-1β were identified. The TNF-α and IL-1β fragments were recovered and subcloned into pPow expression vector digested with *Eco*RI and *Bam*HI.

Expression of rbTNF-α and rbIL-1β. SDS-PAGE and Western blot analysis of proteins from culture supernatant (extracellular) and cellular lysates (intracellular) from *E. coli* DH5α, harboring pPow carrying the TNF-α or IL-1β cDNA insert, were used to identify rbTNF-α and rbIL-1β. Results from SDS-PAGE showed that pPow carrying the TNF-α or IL-1β insert expressed additional proteins in the cellular lysates after heat induction (data not shown). By contrast, the native pPow showed the same protein profile regardless of heat induction. Protein species migrating at the same position as that of the purified 17-kDa rbTNF-α used as a positive standard was visible in SDS-polyacrylamide gels in cell lysates from *E. coli* transformed with pPow carrying the TNF-α cDNA insert after heat induction. In Western blots, monospecific anti-bovine TNF-α and anti-bovine IL-1β antibodies strongly recognized the 17-kDa rbTNF-α and rbIL-1β proteins (Fig. 2 and 3). By

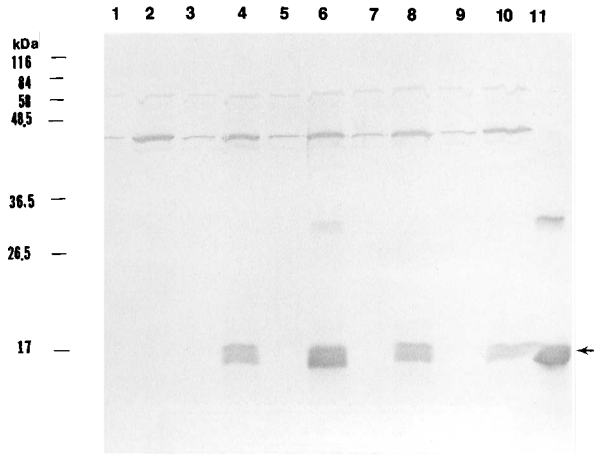

FIG. 2. Western blot analysis of whole cellular proteins from four different *E. coli* DH5α transformants harboring pPow carrying the TNF-α cDNA insert and one transformant harboring native pPow probed with antibodies against rbTNF-α (anti-bovine TNF-α). Lanes: 1 and 2, whole cellular proteins from *E. coli* transformed with native pPow before and after heat induction, respectively; 3, 5, 7, and 9, whole cellular proteins from four different *E. coli* transformants with pPow carrying TNF-α cDNA without heat induction; 4, 6, 8, and 10, whole cellular proteins from the same transformants as those in lanes 3, 5, 7, and 9, but after heat induction; 11, purified rbTNF-α. The arrow indicates the position of the 17-kDa rbTNF-α. The 34-kDa bands in lanes 6 and 11 most likely represent dimeric rbTNF-α. Other immunoreactive bands visible in lanes 1 to 10 are the result of reactivities of various *E. coli* antigens with corresponding antibodies present in rabbit anti-bovine TNF-α. Molecular sizes (in kilodaltons) are indicated on the left.

contrast, preimmune rabbit serum and chicken serum showed no reactivity with these cytokines (data not shown). The polyclonal anti-bovine TNF-α dose dependently inhibited TNF-α bioactivity, while preimmune rabbit serum did not inhibit the

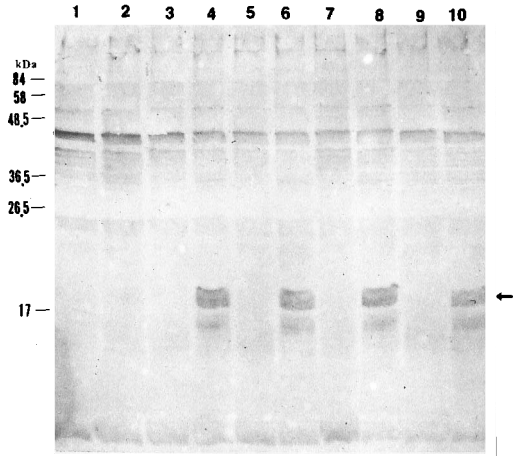

FIG. 3. Western blot analysis of whole cellular proteins from four different *E. coli* DH5α transformants harboring pPow carrying the IL-1β cDNA insert and one transformant harboring native pPow probed with antibodies against IL-1β (anti-bovine IL-1β). Lanes: 1 and 2, whole cellular proteins from *E. coli* transformed with native pPow before and after heat induction, respectively; 3, 5, 7, and 9, whole cellular proteins from four different *E. coli* transformants with pPow carrying IL-1β cDNA without heat induction; 4, 6, 8, and 10, whole cellular proteins from the same transformants as those in lanes 3, 5, 7, and 9, but after heat induction. The arrow indicates the position of a 17-kDa rbIL-1β band that reacted with anti-IL-1β. The other faint bands visible in lanes 1 to 10 are the result of reactivities of various *E. coli* antigens with corresponding antibodies present in anti-bovine IL-1β. Molecular sizes (in kilodaltons) are indicated on the left.

TABLE 2. Comparison of extracellular cytokine concentrations in culture supernatants from *E. coli* DH5α transformed with pPow carrying TNF-α or IL-1β cDNA or from *E. coli* with native pPow[a]

Transformants	Concn (pg/ml) of[b]:	
	rbTNF-α	rbIL-1β
E. coli with pPow (with TNF-α or IL-1β cDNA insert)		
Clone 1	9.035 ± 3.778	0.6023 ± 0.1205
Clone 2	8.088 ± 1.631	0.6683 ± 0.2320
E. coli with native pPow only	0.728 ± 0.253	0.0018 ± 0.0003

[a] Determined by bioassays as described in Materials and Methods.
[b] Data represent the means of duplicate determinations from three independent experiments.

bioactivity in the culture supernatant. Additional studies showed that the expression of rbTNF-α as shown by Western blot staining was predominantly intracellular, although some extracellular expression was noticed with TNF-α (data not shown). While the expression of rbIL-1β was also intracellular, small amounts were also expressed extracellularly as shown by the reactivity of a protein in the culture supernatant with anti-IL-1β seen as a faint band in the Western blot (data not shown). We performed cytokine bioassays with the sterile culture supernatants from *E. coli* DH5α transformed with pPow carrying TNF-α or IL-1β cDNA inserts. For comparison, culture supernatant from *E. coli* transformed with the native vector pPow was subjected to the same bioassays. As shown in Table 2, TNF-α bioactivity was present only in the culture supernatant from *E. coli* transformed with pPow carrying the TNF-α insert and not in the culture supernatant from the vector control. Specificity was controlled by the ability of anti-bovine TNF-α antibodies to neutralize the cytotoxic activity of supernatant on WEHI 164 clone 13 cells (data not shown). Also, culture supernatant from *E. coli* DH5α transformed with pPow carrying the IL-1β insert and not that with the native vector showed IL-1β bioactivity (Table 2). The presence of authentic IL-1β was confirmed by the ability of IL-1ra to neutralize the bioactivity in a concentration-dependent manner, and at concentrations of 100 ng/ml or higher, it inhibited the bioactivity by 100%.

Kinetics of TNF-α and IL-1β mRNA expression in AMs stimulated with *P. haemolytica* LPS. To determine the concentration of LPS from *P. haemolytica* which would induce maximal expression of TNF-α and IL-1β mRNAs, AMs were exposed to 0.01 to 10 μg of LPS per ml for 4 h. Both TNF-α and IL-1β mRNAs were expressed at a concentration threshold of 0.01 μg/ml, with maximum expression at LPS concentrations of 1 to 10 μg/ml (Fig. 4) and no additional mRNA induction at higher concentrations (data not shown). However, in the absence of LPS, neither TNF-α- nor IL-1β-specific mRNA could be detected by Northern blot analysis. Resting AMs did not contain any detectable TNF-α or IL-1β mRNA. In subsequent experiments, 1 μg of *P. haemolytica* LPS per ml was used for stimulation of bovine AMs. As shown in Fig. 5, LPS-stimulated AMs expressed TNF-α and IL-1β mRNA levels in a time-dependent fashion. Maximal levels of TNF-α and IL-1β mRNA expression were seen at 1 to 2 h after stimulation and then declined steadily up to 16 h, and expression was undetectable by 24 h. The levels of expression of TNF-α and IL-1β mRNAs were inhibited by polymyxin B (1 or 10 μg/ml) by 95 and 90%, respectively (Fig. 6). Approximately the same magnitudes of inhibition were seen with AMs either preincubated

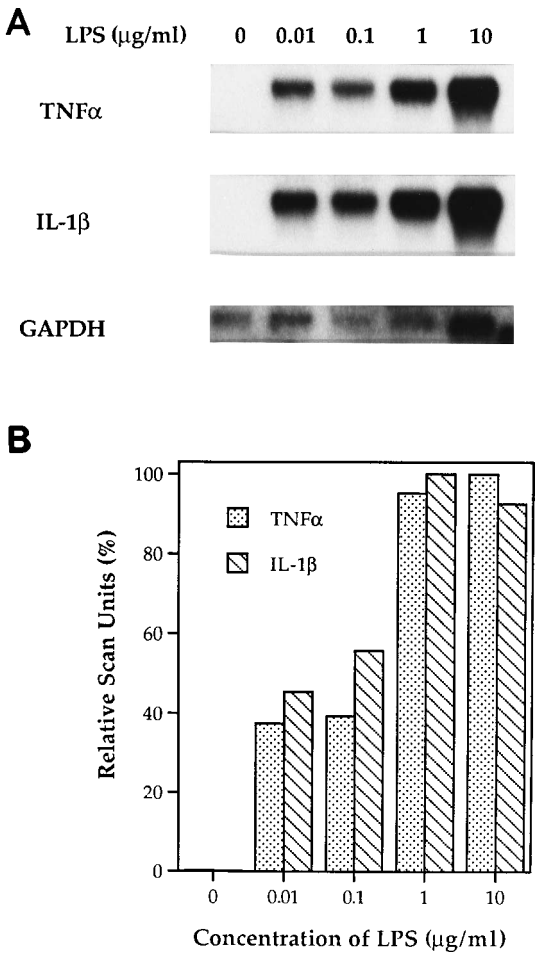

FIG. 4. Dose dependence of bovine TNF-α and IL-1β mRNA expression in bovine AMs stimulated with various concentrations of LPS from *P. haemolytica* 12296 for 4 h. (A) Northern blot analysis with TNF-α and IL-1β probes was performed as described in Materials and Methods. (B) Relative levels of TNF-α and IL-1β mRNA were quantitated by densitometric scanning and normalized to glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA levels. The data depicted are representative of three individual experiments.

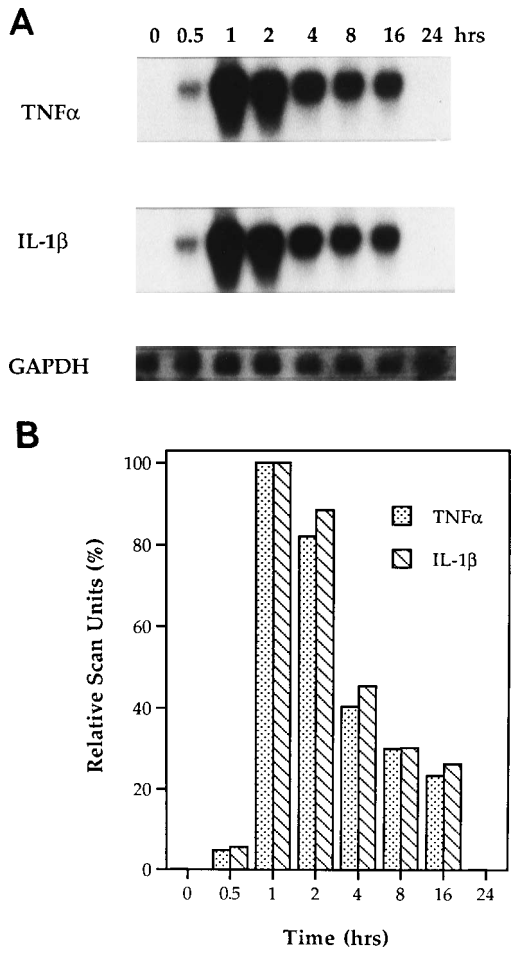

FIG. 5. Time course of bovine TNF-α and IL-1β mRNA expression. Bovine AMs were cultured in the presence of 1 μg of LPS from *P. haemolytica* 12296 per ml for the indicated lengths of time. (A) Total RNA was extracted from the cells and subjected to the Northern blot analysis as described in the Materials and Methods section. (B) Relative levels of TNF-α and IL-1β mRNA were quantitated by densitometric scanning and normalized to glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA levels. The data shown are representative of three individual studies.

with polymyxin B before addition of LPS or exposed to polymyxin B and LPS at the same time.

Kinetics of secretion of bioactive TNF-α and IL-1 from AMs stimulated with *P. haemolytica* LPS. LPS-stimulated AMs secreted TNF-α in a dose-dependent fashion which peaked at 1 μg/ml. By contrast, there were lesser amounts of IL-1 present in the conditioned medium from AMs stimulated with 1 μg of LPS per ml than those with 0.1 μg and 10 μg of LPS per ml (Fig. 7). This decline in concentration of IL-1 may be due to the presence of inhibitors in the AM-conditioned medium. To examine the relationship between TNF-α and IL-1β mRNA transcription and secretion of TNF-α and IL-1 proteins, conditioned media from AMs stimulated with 1 μg of *P. haemolytica* LPS per ml were collected at various times and assessed for bioactivity. The results, shown in Fig. 8, revealed that production of TNF-α was observed at 1 h, peaked at 4 h, and dropped significantly by 8 h. By contrast, production of IL-1 was observed at 4 h, continued to increase, and peaked at 24 h. Anti-bovine TNF-α antibodies and IL-1ra neutralized the biological activities of TNF-α and IL-1, respectively, present in conditioned medium. Furthermore, polymyxin B at a concentration of 1 or 10 μg/ml inhibited LPS-induced TNF-α and IL-1 production by approximately 87 and 95%, respectively. A noteworthy observation was that polymyxin B alone had significant cytotoxicity for WEHI cells and D10 (N4) cells at 100 μg/ml, intermediate toxicity at 25 μg/ml, and no effect at 1 to 10 μg/ml.

DISCUSSION

The AMs are a major cell population in alveolar spaces and play an important role in the defense against respiratory tract infections by microorganisms. The activation and release of bacteriocidal substances from the AMs are essential for fighting infection. However, if AM activation is not tightly regulated, excessive production of inflammatory cytokines is deleterious and can induce damage to the lung tissues. Indeed, the results of several recent investigations (7, 17, 18, 27, 33, 37) have suggested that certain inflammatory cytokines, i.e., TNF-α, IL-1β, IL-6, and IL-8, play a critical role in the pathogenesis of septic shock lung injury. Although the pathogenesis of bovine pneumonic pasteurellosis is undoubtedly complex and not well understood, several observations suggest that

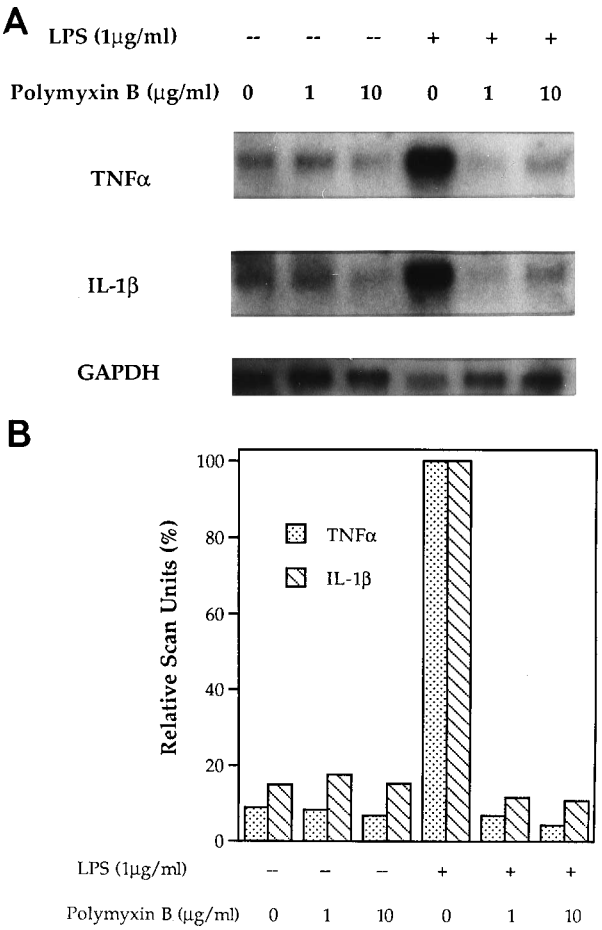

FIG. 6. Effects of polymyxin B on expression of TNF-α and IL-1β mRNA. Bovine AMs were cultured in the presence of 1 μg of LPS from *P. haemolytica* 12296 per ml with and without preincubation with polymyxin B at 0, 1, and 10 μg/ml. (A) After 4 h, RNA was isolated and a Northern blot was performed as described in Materials and Methods. (B) Relative levels of TNF-α and IL-1β mRNA were quantitated by densitometric scanning and normalized to glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA levels. This experiment was repeated four times with similar results.

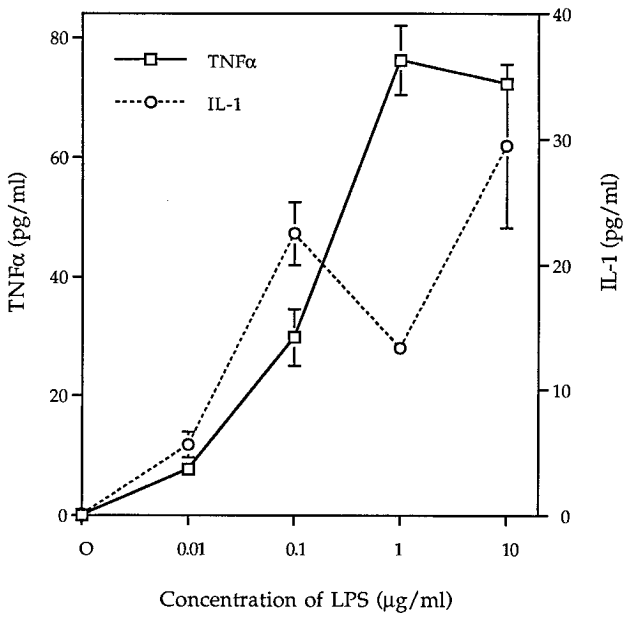

FIG. 7. Dose-dependent production of extracellular TNF-α and IL-1 from LPS-stimulated bovine AMs as assessed by bioassays. AMs were cultured with the indicated concentrations of LPS from *P. haemolytica* 12296 for 4 h, and conditioned media were subjected to bioassays. Bioactivities were obtained from the means of triplicate wells and expressed in picograms per milliliter as described in Materials and Methods. The values represent the means ± standard deviations from three independent experiments.

AMs play a significant role in mediating the inflammatory response in the lung (42, 43). However, the role of AM-derived inflammatory cytokines, particularly TNF-α and IL-1β, in the pathogenesis of lung injury has not been defined. To initiate studies of this, we required the use of TNF-α and IL-1β cDNAs as probes for Northern blot analysis. Since these were not readily available, we decided first to isolate the bovine TNF-α and IL-1β cDNAs from an LPS-stimulated bovine AM cDNA library by use of the PCR method. The PCR-amplified fragments of 488 and 474 bp, which coded for the mature protein portions of bovine TNF-α and IL-1β, were first cloned into pBluescript KS(+) and then subcloned into pPow. Comparison of restriction enzyme analysis patterns of these cloned fragments in pBluescript KS(+) with previous reports (9, 22) confirmed that they were indeed bovine TNF-α and IL-1β cDNAs. Data from SDS-PAGE and Western blot analysis of extracellular and intracellular proteins from *E. coli* DH5α transformed with pPow carrying the TNF-α or IL-1β cDNA insert clearly indicated the presence of these cytokines, which specifically reacted in Western blots with anti-bovine TNF-α and anti-bovine IL-1β antibodies, respectively. The same extracellular proteins from *E. coli* DH5α also showed bioactivity for TNF-α or IL-1β, and their activities were neutralized by anti-bovine TNF-α antibodies or recombinant human IL-1ra.

By using the cloned cDNAs as probes in Northern blots, we investigated the kinetics of TNF-α and IL-1β mRNA expression in bovine AMs stimulated with purified LPS from *P. haemolytica* 12296. The results of this study showed that TNF-α and IL-1β mRNA expression in bovine AMs was sensitive to induction by LPS from *P. haemolytica*. The effect of LPS on TNF-α and IL-1β expression was dose dependent, and induction was observed at a concentration of 0.01 μg/ml (Fig. 4). Northern blot analysis also showed that the TNF-α and IL-1β message was regulated in a time-dependent manner after stimulation with 1 μg of LPS per ml. Induction of TNF-α and IL-1β mRNA species, which were approximately 2.0 kb in size, occurred as early as 0.5 h poststimulation, with maximum levels of the transcript at 1 to 2 h and undetectable levels by 24 h (Fig. 5). Secreted TNF-α measured by bioassay peaked at 4 h and was produced in lesser concentrations in conditioned medium throughout the 24 h (Fig. 8). By contrast, release of bioactive IL-1 was observed at 4 h and continued to accumulate in higher concentrations up to 24 h. By utilizing anti-bovine TNF-α antibodies and human recombinant IL-1ra, the specificities of these bioactivities present in LPS-stimulated AM conditioned media were confirmed as bovine TNF-α and IL-1, respectively. In addition, polymyxin B abrogated the ability of LPS to induce expression (Fig. 6) and production of TNF-α and IL-1β in AMs. Thus, the LPS from *P. haemolytica* appears to be a specific and direct inducer of TNF-α and IL-1β in bovine AMs.

In related studies, maximal expression of TNF-α mRNA was seen at 2 h and disappeared by 24 h after stimulation of porcine and ovine AMs with LPS from *E. coli* and *Salmonella abortus equi*, respectively (5, 14, 28). Other studies suggest that maximal expression of IL-1β mRNA in porcine and ovine AMs

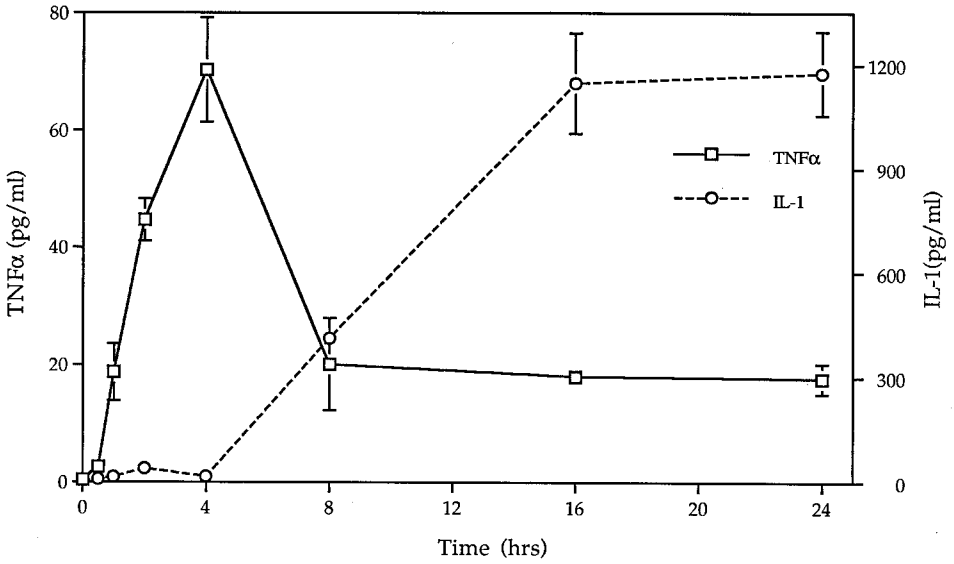

FIG. 8. Time-dependent production of extracellular TNF-α and IL-1 by bovine AMs cultured in the presence of 1 μg of LPS from *P. haemolytica* 12296 per ml for the indicated lengths of time, as assessed by bioassays. The bioactivity values used to produce the figure were prepared as described in the legend to Fig. 7. The plots depict values which are means ± standard deviations from three different experiments.

occurred at 6 to 8 h and 4 h, respectively, after stimulation with LPS from *E. coli* (13, 15). Furthermore, ovine (13) and porcine (15) IL-1β gene expression declined at 8 h and disappeared by 24 h. However, our data showed a much earlier maximal expression of TNF-α and IL-1β mRNAs at 1 to 2 h postinduction in bovine AMs exposed to LPS from *P. haemolytica*. This earlier gene expression in bovine AMs in comparison with that of porcine and ovine AMs appears to be due to differences in cell species sensitivity and to responses to different LPSs. This is a plausible explanation because more recent studies have documented that LPSs derived from different bacterial species may react with different receptors on macrophages and are activated by different signal transduction pathways (23, 34). In a more recent study (16), IL-1β mRNA was detected by use of the reverse transcription-PCR at 3 to 24 h in poly(A)$^+$ RNAs isolated from bovine peripheral blood mononuclear cells stimulated with LPS from *E. coli*. Although the reverse transcription-PCR is more sensitive than Northern blot analysis, no attempts were made in this study to detect gene expression at an earlier time.

In ruminants, several observations point to an important role for TNF-α in septic shock that was once ascribed to endotoxin. First, when cattle are injected with rbTNF-α, clinical signs suggestive of septic shock similar to those occurring in other species appeared (25). Moreover, the presence of circulating levels of TNF-α in sera from endotoxemic calves may suggest a role of TNF-α in endotoxemia in ruminants (2). Other investigators (1) have reported that bovine peripheral blood monocytes and AMs are capable of releasing TNF-α following stimulation with bacterial LPS and that receptors for TNF-α are present on bovine macrophages, lymphocytes, and neutrophils (24).

Recently, Pace et al. (26) described the elevation of serum TNF-α levels in calves following *P. haemolytica*-induced experimental pneumonic pasteurellosis. More importantly, the elevated TNF-α levels paralleled the development of clinical signs and pneumonic lesions. Results from our in vitro experiments have established that bovine AMs are a major source of TNF-α and IL-1β in response to LPS from *P. haemolytica*. Taken together, these results suggest that inflammatory cytokines such as TNF-α and IL-1β may play an important role in the pathogenesis of lung injury in pneumonic pasteurellosis. However, additional studies showing the presence of inflammatory cytokine expression with lung lesions must be completed before we can establish undoubtedly a direct role for these cytokines in the pathogenesis of lung injury in bovine pneumonic pasteurellosis.

ACKNOWLEDGMENTS

We thank Charles J. Czuprynski from the Department of Pathobiological Sciences, University of Wisconsin, Madison, for his suggestions in the course of synthesis of appropriate upstream and downstream primers for PCR. We are grateful to Mark S. Rutherford for critical reading of the manuscript.

This research was supported by USDA-NRI competitive grant 91-37204-6409 (to S.K.M.).

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