INFECTION AND IMMUNITY, Sept. 1995, p. 3587–3594
0019-9567/95/$04.00+0
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Vol. 63, No. 9

Inflammatory Cytokine Expression in Swine Experimentally Infected with *Actinobacillus pleuropneumoniae*

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Received 22 July 1994/Returned for modification 22 November 1994/Accepted 12 June 1995

An *Actinobacillus pleuropneumoniae* infection model in swine was established to study the expression of inflammatory cytokines during acute respiratory disease. Lavage fluid, lavage cells consisting primarily of alveolar macrophages, and lung tissue were analyzed for the presence of various cytokines at 2, 4, 8, and 24 h following endotracheal inoculation of *A. pleuropneumoniae*. Interleukin-1β (IL-1) and IL-8 mRNA levels were elevated within 2 h in lavage cells of animals inoculated with *A. pleuropneumoniae* but not in cells from controls treated with saline-bovine serum albumin, based on Northern (RNA blot) analysis. Tumor necrosis factor (TNF) mRNA was present at low levels in all animals, and the level was not increased at any time point. In situ hybridization was more sensitive than Northern blotting and revealed elevations of all three cytokines in lavage cells within 2 to 4 h of *A. pleuropneumoniae* inoculation. IL-6 was detected in lavage cells by in situ hybridization but not by Northern blotting. In lung tissue obtained 18 to 24 h after *A. pleuropneumoniae* instillation, all cytokine mRNAs, including that of IL-6, were detected by Northern blot analysis. The levels of bioactive IL-1 and IL-6 in lavage fluids increased approximately 1,000-fold following *A. pleuropneumoniae* inoculation, but TNF bioactivity was not detected. Morphological localization of cytokine mRNAs by in situ hybridization indicated markedly increased levels of TNF, IL-1, and IL-8 mRNAs at the periphery of focal lung lesions. These findings indicate that inflammatory cytokines, particularly IL-1 and IL-8, are associated with the development of pleuropneumonia and may contribute to disease severity.

Actinobacillus pleuropneumoniae is the etiological agent of porcine pleuropneumonia, a severe respiratory disease of swine which is characterized by a necrotizing fibrinous pneumonia and pleuritis. Several bacterial components, including lipopolysaccharide (LPS), toxins, and polysaccharide capsule, likely contribute in combination to the disease (19, 29). It appears that no single bacterial factor is both necessary and sufficient for disease, since no single mutant defective in one or another factor is nonpathogenic (30), nor does antibody neutralizing one or another activity provide complete protection from the organism (7, 10, 28). The complex nature of porcine pleuropneumonia has translated into a lack of efficacious methods for treatment and prevention and emphasizes the need for a better understanding of its pathogenesis.

One of the early events in acute inflammation of the lung is the release of immune modulators, including the inflammatory cytokines tumor necrosis factor alpha (TNF), interleukin-1 (IL-1), IL-6, and IL-8. TNF, IL-1, and IL-8 play important roles in respiratory disease pathogenesis in models of sepsis-induced lung injury. For example, bacterial endotoxins in the circulation cause hypotension, shock, and death, but anti-TNF antibodies and IL-1 receptor antagonist can prevent lethal shock (3, 24, 27, 36, 39). Furthermore, a combination of TNF and IL-1 accentuates lung tissue destruction, in part by mutual autocrine and paracrine upregulation (6). TNF-induced lung injury also can be abrogated by depletion of neutrophils (34). This finding suggests that TNF may not be directly responsible for sepsis-induced tissue damage, but rather is an inducer of the neutrophil chemoattractant IL-8. IL-8 may be a key mediator of lung damage because of its ability to act as a potent chemokine which recruits degranulating neutrophils to the lung (20).

Although antibodies against IL-6 can increase survival during *Escherichia coli* septicemia, the importance of IL-6 in sepsis-induced respiratory pathogenesis is not well understood (33). It is clear, however, that IL-6 has an important function in the induction of the acute-phase response and acute-phase proteins, which may serve as predictors of stress and disease (12, 13, 31).

The alveolar macrophage encounters bacteria early in a respiratory disease insult and readily expresses TNF, IL-1, and IL-8 in response to bacterial LPS (2, 18, 21). Overproduction of inflammatory cytokines within the lung may exacerbate the direct effects of *A. pleuropneumoniae* and lead to host-mediated tissue damage. The purpose of this study was to characterize the patterns of inflammatory cytokine expression in the lungs of pigs following *A. pleuropneumoniae* infection. Thus, we examined lung lavage fluid, lavage cells, and lung tissue for the presence of TNF, IL-1, IL-6, and IL-8 mRNAs by Northern (RNA) blotting and in situ hybridization and determined levels of TNF, IL-1, and IL-6 bioactivity in lavage fluid. Significant elevations in inflammatory cytokine levels were observed in the airways and in lung tissue of *A. pleuropneumoniae*-infected animals. Furthermore, TNF, IL-1, and IL-8 mRNA expression was specifically localized to the periphery of pleuropneumonic lesions. These findings suggest that inflammatory cytokines may contribute to the development and/or progression of porcine pleuropneumonia.

MATERIALS AND METHODS

Animals. Conventional cross-bred pigs, 6 to 8 weeks old, were housed in isolation facilities according to University of Minnesota Institutional Animal Care and Use Committee guidelines. Animals were derived from two different

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source herds and were seronegative for *A. pleuropneumoniae* as determined by the Iowa State University Veterinary Diagnostic Laboratory, Ames, Iowa.

Bacteria. All in vivo inoculations were performed with *A. pleuropneumoniae* serotype 1, strain L91-2, a field strain obtained from an outbreak in Iowa. Logarithmically growing bacteria were used to prepare inocula for animals. Cultures of *A. pleuropneumoniae* were grown in 3% tryptic soy broth containing 1% NZ amine, 0.1% yeast extract, and 0.001% NAD (Sigma Chemical Co., St. Louis, Mo.). Unless otherwise indicated, the components used for bacterial media were obtained from Difco Laboratories, Detroit, Mich.

A low-virulence *A. pleuropneumoniae* culture was produced by serial in vitro passage of *A. pleuropneumoniae* serotype 1 strain L91-2 on solid growth medium. Single colonies were passaged approximately four times, until atypical morphology was observed. Colonies were creamier in appearance and two to three times larger and grew more rapidly than nonpassaged organisms. A broth culture was then started, and inocula were prepared as described for the field strain.

Heat-killed bacteria were prepared by heating serotype 1 organisms at 80°C for 15 min before inoculation. Tenfold serial dilutions of heat-treated inocula were plated on Casman's agar in triplicate to confirm nonviability.

Experimental infection protocol. *A. pleuropneumoniae* cells were washed once with phosphate-buffered saline (PBS, pH 7.4) and diluted to a final A_{540} of 0.1 (approximately 5×10^7 CFU/ml). The washed cells were then diluted in PBS containing 5% bovine serum albumin (PBS/BSA) to approximately 10^4 CFU/ml. Pigs were anesthetized intramuscularly with 2.5 mg of xylazine and 12 mg of ketamine HCl per kg. Five milliliters of a 10^4-CFU/ml bacterial culture or of PBS/BSA was inoculated endotracheally to yield an inoculum of approximately 5×10^4 CFU per pig. Bacterial plate counts were performed immediately before and after experimental infection to determine viable-cell counts. Animals were killed at various times after inoculation by anesthetizing them with ketamine and xylazine and then giving them a barbiturate overdose.

Bioassays. The L929 cytotoxicity assay for detection of porcine TNF was described in detail previously (2). Briefly, L929 mouse fibroblasts were seeded into 96-well plates, and after overnight incubation at 37°C and 5% CO_2, serial twofold dilutions of the test samples were added to the plates. All samples were tested in duplicate. After approximately 20 h of incubation at 37°C, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was added to all wells, and the cells were incubated for an additional 2.5 h at 37°C. The supernatants were discarded, and formazan crystals were solubilized in 100 μl of 50% dimethylformamide–20% sodium dodecyl sulfate (SDS) (pH 4.7) per well. Color development was quantitated at 570 nm with a Molecular Devices enzyme-linked immunosorbent assay reader (Menlo Park, Calif.). Human recombinant TNF (Knoll Pharmaceuticals, Whippany, N.J.) was used as a positive control for cytotoxicity. Percent cytotoxicity was determined from the mean of duplicate wells as previously reported (2).

IL-1 was measured by using the D10(N4)M murine T-cell line as previously reported (17). Cells were maintained in RPMI 1640 containing 10% fetal calf serum (FCS), 3 μg of concanavalin A per ml, 20 ng of recombinant human IL-2 (R & D Systems, Minneapolis, Minn.) per ml, and 40 pg of recombinant human IL-1 (R & D Systems) per ml. Lavage samples were added at various dilutions in duplicate to 96-well culture plates containing 10^4 cells which had been washed free of exogenous IL-1 per well. Cultures were allowed to incubate for 3 days at 37°C. Proliferation was measured by the addition of MTT, and the plates were incubated for an additional 3 h. Formazan crystals were solubilized as described above for the L929 assay and quantitated at 570 nm. The proliferative responses were transformed into picograms per milliliter by interpolation based on a standard curve with recombinant human IL-1.

Bioactive IL-6 was determined in a B9 hybridoma proliferation assay as previously described (1). Cells were maintained in RPMI 1640 containing 2% FCS and supplemented with 1 ng of recombinant mouse IL-6 (R & D Systems) per ml. Lavage samples were added at various dilutions in duplicate to 96-well culture plates containing 10^4 B9 cells which had been washed free of exogenous IL-6 per well. After 3 days of incubation at 37°C, MTT was added to each well, and the incubation was continued for an additional 4 h. Formazan crystals were solubilized as described above, and the optical density at 570 nm was determined. One unit of activity was defined by a standard porcine IL-6-containing conditioned medium sample as the amount that stimulated a half-maximal B9 proliferative response.

RNA isolation and Northern blotting. Total RNA was isolated from lung tissue or lavage cells and solubilized in guanidine isothiocyanate as previously described (8). RNA denaturation and electrophoresis in 0.7% agarose–2.2 M formaldehyde gels were carried out as previously described (2). Sample RNA loading equivalency was monitored by the addition of 1 μl of 0.1% ethidium bromide per gel lane, followed by visualization of the RNA in gels by UV transillumination. RNA was transferred to nylon membranes (Micron Separations Inc., Westborough, Mass.) in 20× SSC buffer (1× SSC contains 150 mM NaCl and 15 mM sodium citrate [pH 7.0]). Transferred nucleic acids were UV fixed to membranes, and prehybridization was carried out for 30 min at 42°C. Prehybridization and hybridization buffers consisted of 0.25 M phosphate buffer, pH 7.4, containing 1 mM EDTA, 7% SDS, and 50% formamide (9). Hybridization was allowed to proceed overnight at 42°C. Membranes were washed to a stringency of 0.1× SSC–0.1% SDS at 60°C. Membranes were autoradiographed overnight at −70°C with Kodak XAR film and two intensifier screens.

Northern blotting probes. All probes used in Northern blotting were random primed with [^{32}P]dCTP to a specific activity of 5×10^8 to 10×10^8 cpm/μg of DNA. The following porcine cytokines were cloned from a λgt10 cDNA library and used to derive the probes used for Northern blotting: TNF (EMBL accession X57321), IL-1β (GenBank M86725), IL-6 (GenBank M86722), and IL-8 (GenBank M86923). A probe specific for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was obtained from William A. Toscano (Tulane University, New Orleans, La.) and used to monitor lane loadings and transfer efficiency.

In situ hybridization probes. Antisense oligonucleotides complementary to bases 573 to 607 of porcine TNF (35 bases [b], 5′-ATGGTGTGAGTGAGGAA AACGTTGGTGGAAGGGCA-3′), bases 354 to 388 of IL-1β (35 b, 5′-TGCAC GTTTCAAGGATGATGGGCTCTTCTTCAAAG-3′), bases 358 to 392 of IL-6 (35 b, 5′-GTCTCCTGATTGAACCCAGATTGGAAGCATCCGTC-3′), and bases 201 to 248 of IL-8 (48 b, 5′-AATCACTCTCAGTTCCTTGATAAATTT GGGGTGGAAAGGTGTGGAATG-3′) were synthesized on an Applied Biosystems 391 DNA Synthesizer (Foster City, Calif.) according to porcine sequences determined from previously cloned cDNAs (see Northern blotting probes). Oligo(dT)$_{30}$ was synthesized and used as a positive control probe, and the sense strand of IL-8 complementary to the antisense oligonucleotide was synthesized and used as the negative control probe. All oligonucleotides were 3′-end digoxigenin tailed with the Genius system as described by Boehringer Mannheim (5). Probes were used at a final concentration of 1.5 pmol per tissue section in a total volume of 30 μl.

In situ hybridization in cells and tissue. Approximately 0.5-cm^3 blocks of lung tissue were fixed in paraformaldehyde-lysine-periodate buffer (PLP) (23) for approximately 18 h and stored in 70% ethanol until paraffin embedment. Three regions, representing the apical, middle, and caudal lobes, were sampled. The remaining lung not used for tissue collection was lavaged with PBS, and the fluid was collected for total RNA isolation and Northern blotting as described above. In addition, a small portion of lavage fluid was spotted onto slides, air dried, fixed in PLP for 5 min, rinsed in 70% ethanol, and stored under 70% ethanol at −20°C until hybridization was performed. All slides used for in situ hybridization were treated with 1% *N*-(β-aminoethyl)-γ-aminopropyltrimethoxysilane (Pierce, Rockford, Ill.), pH 3.45, to enhance the adherence of tissues and cells to slides (22).

Sections (6 μm) were cut from paraffin-embedded tissues and dried in a 37°C oven overnight before hybridization or prior to storage at −20°C. After drying, sections were dewaxed in two xylene dips each for 5 min and rehydrated through successive 2-min treatments in 90, 70, and 50% ethanol. Sections were then incubated for 20 min in 0.2 M HCl, acetylated in 0.1 M triethanolamine (pH 8.0) containing 0.5% acetic anhydride for 10 min, and rinsed in 0.2 M Tris (pH 7.4) containing 0.1 M glycine for 10 min. All treatments were performed at room temperature.

Prior to prehybridization, sections were incubated in 2× SSC at 70°C for 10 min. The slides were placed flat in a humidified chamber, and approximately 100 μl of prehybridization buffer per slide was added for 30 min at 37°C. Prehybridization buffer consisted of 50% formamide, 4× SSC, 1× Denhardt's solution (50× Denhardt's solution consists of 1% Ficoll 400, 1% polyvinylpyrrolidone 360, and 1% BSA), and 10% dextran sulfate. All reagents were purchased from Sigma Chemical Co. After prehybridization, excess solution was gently blotted from the slides, and 30 μl of prehybridization buffer containing the appropriate probe was applied and covered with a small piece of parafilm. Hybridizations were allowed to proceed for approximately 16 h in a humidified chamber at 37°C. Slides were then washed successively in 4× SSC, 2× SSC, and 0.2× SSC at 37°C. Each wash was performed twice for 5 min with gentle agitation. In situ hybridization of lavage cell slides was conducted as stated above except that the xylene dewaxing and HCl treatments were omitted. Immunological detection was carried out as described by Boehringer Mannheim (4). This system used an antidigoxigenin antibody conjugated to alkaline phosphatase and a nitroblue tetrazolium substrate to produce a dark blue-purple precipitate over positively staining cells.

Quantitation and statistical analysis. Northern blotting experiments were quantitated with an AMBIS 4000 image analyzer. The levels obtained with cytokine probes were normalized to GAPDH expression and compared with the maximal expression obtained for IL-8. Relative values of mRNA expression, percent in situ hybridization-positive cells, and bioassay results were analyzed for statistical significance by analysis of variance, and P values of ≤ 0.05 were considered significant. Individual differences between groups and interactions among groups were determined by Fisher's protected test of least significant differences.

RESULTS

Kinetics of cytokine mRNA expression in lavage fluid cells. In order to examine the time course of inflammatory cytokine expression in the lungs of experimentally infected pigs, animals were inoculated endotracheally with PBS/BSA with or without 5×10^4 CFU of *A. pleuropneumoniae*. At 0, 2, 4, 8, and 24 h after inoculation, three infected and one control animal were killed. Clinical signs, gross pathology, histopathology, and bac-

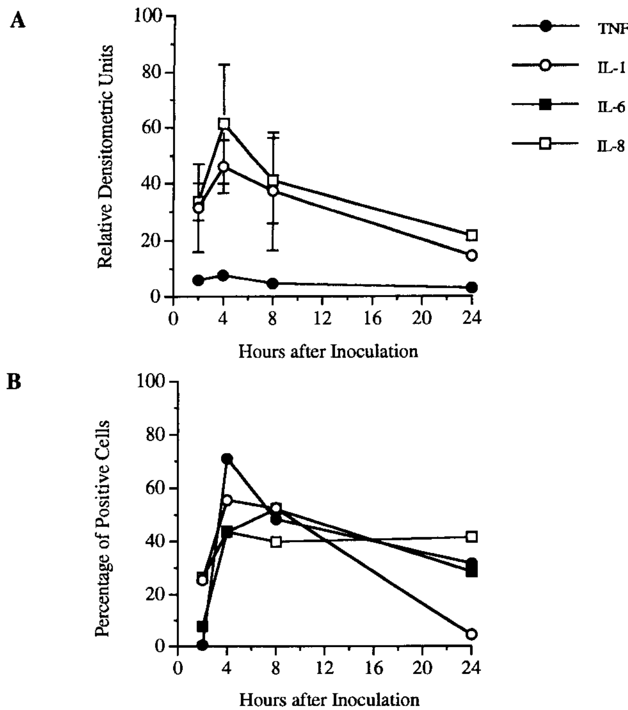

FIG. 1. Time course of cytokine mRNA expression following *A. pleuropneumoniae* challenge in total RNA obtained from lung lavage fluid cells. (A) Northern blotting results were quantified as described in Materials and Methods and are shown as the mean ± SE for three animals at 2, 4, or 8 h or two animals at 24 h after *A. pleuropneumoniae* inoculation. IL-6 was not detected by Northern blotting. (B) In situ hybridization results represent the mean percent positive cells for the same animals in panel A. SEs were omitted from the data in panel B for clarity but are equivalent to those in panel A.

terial reisolations were consistent with pleuropneumonia in animals inoculated with *A. pleuropneumoniae* (unpublished data). No evidence of disease was observed in uninfected controls.

Control uninfected lavage cell populations were >96% macrophages, as determined previously by nonspecific esterase staining (2). Within 2 h of endotracheal inoculation with *A. pleuropneumoniae*, the percentage of macrophages in the lavage fluid appeared to decrease, as determined by Wright stain morphology. Similar observations of cell composition from bronchoalveolar lavage samples from pneumonic pigs were reported previously (15). However, repeated attempts to accurately identify and quantify the percentage of neutrophils in lavage fluids collected from pigs endotracheally inoculated with virulent *A. pleuropneumoniae* were unsuccessful. Cell morphology was consistently poor, presumably because of the presence of bacterial cytotoxin, and accurate determinations were not possible with Wright or nonspecific esterase stains.

Total RNA was obtained from freshly isolated lung lavage cells, and levels of TNF, IL-1, IL-6, and IL-8 expression were evaluated by Northern blotting. The results indicated that *A. pleuropneumoniae* infection induced a rapid increase in IL-1 and IL-8 cytokine mRNA levels compared with levels in uninfected controls as early as 2 h after bacterial inoculation (Fig. 1A). Low levels of TNF were detected in both *A. pleuropneumoniae*- and PBS/BSA-inoculated pigs throughout the 24-h period. IL-1 and IL-8 mRNA levels remained elevated above that of controls for at least 24 h (Fig. 1A). The IL-1 and IL-8 mRNA levels observed in PBS/BSA-treated control pigs were very similar to those shown for TNF in Fig. 1A. The mean

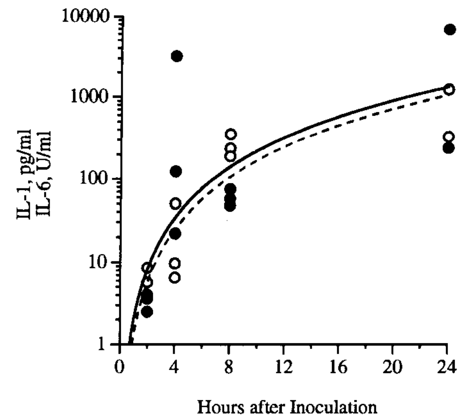

FIG. 2. Accumulation of IL-1 (●) and IL-6 (○) bioactivity in lung lavage fluid collected from three animals at various times after *A. pleuropneumoniae* inoculation. IL-1 and IL-6 activities were not detected in lavage fluids from PBS/BSA-inoculated animals.

densitometric values for the 2, 4, 8, and 24 h time points for IL-1 and IL-8 in animals inoculated with PBS/BSA were 5 ± 1.5 and 8 ± 2 (mean standard error [SE]), respectively. IL-6 was not detected in lavage cells by Northern analysis.

Lavage cell smears were also prepared in order to study cytokine expression at the single-cell level and to compare the sensitivity of detection by Northern blotting and in situ hybridization. The in situ hybridization data confirmed the Northern blotting findings with two exceptions. First, the in situ hybridization results suggested that the IL-6 level was also elevated in lung lavage cells from *A. pleuropneumoniae*-infected animals but not in controls within 4 h of infection (Fig. 1B). Second, in situ hybridization detected a dramatic increase in TNF mRNA levels in *A. pleuropneumoniae*-inoculated animals by 4 h after instillation (Fig. 1B), but not in PBS/BSA controls. Essentially no inflammatory cytokine mRNA was found in sham-inoculated pigs by in situ hybridization analyses.

Kinetics of cytokine protein production in lavage fluid. The presence of cytokines in lavage fluids was assessed to correlate cytokine mRNA expression with protein production. A rapid and sustained increase in IL-1 and IL-6 bioactivity was detected in lavage fluid following endotracheal instillation of *A. pleuropneumoniae* but not of PBS/BSA (Fig. 2). IL-1 and IL-6 levels were elevated 2 h after *A. pleuropneumoniae* inoculation and remained high for at least 24 h. No IL-1 or IL-6 activities were detected in lavage fluid from PBS/BSA-inoculated pigs. In contrast to IL-1 and IL-6, no TNF activity was detected in lavage fluids at any time. IL-8 was not measured because a specific bioassay was not available.

To determine if TNF inhibitors in lung lavage fluids of infected animals were masking the production of TNF, a standard dose-response curve for recombinant TNF was performed alone and with 1% lavage fluid from control and infected pigs. Lavage fluids from pigs challenged with virulent *A. pleuropneumoniae* and pigs given PBS/BSA did not suppress L929 cell cytotoxicity at any concentration of TNF from 10 pg to 10 ng per ml (data not shown).

Cytokine expression in lung tissue versus lavage cells. The analysis of cells in lavage fluid focused primarily on inflammatory cytokine expression in alveolar macrophages and infiltrating neutrophils. However, various cell types in lung tissue, including fibroblasts and endothelial cells, may express a variety of cytokines. To compare the pattern of cytokine expression in lung tissue with that in macrophages and neutrophils,

TABLE 1. Northern blot analysis of cytokine mRNA expression in lung tissue and lavage cells 18 h after inoculation[a]

Group	Cytokine (avg ± SE)							
	TNF		IL-1		IL-6		IL-8	
	Lavage	Lung	Lavage	Lung	Lavage	Lung	Lavage	Lung
Control	74 ± 4	3 ± 0.9	10 ± 5	0.2 ± 0.2	0 ± 0	3 ± 2	9 ± 3	6 ± 0
Infected	68 ± 4	18 ± 2[b]	52 ± 9[b]	47 ± 11[b]	0 ± 0	36 ± 9[b]	42 ± 3[b]	44 ± 7[b]

[a] Values represent average relative densitometric units ± SE for four PBS/BSA-inoculated pigs (controls) or nine *A. pleuropneumoniae*-infected pigs, as described in Materials and Methods. Total RNA was isolated from lavage cells or from lung tissue.

[b] Value is significantly different from that for the control group at $P \leq 0.05$, as determined by analysis of variance.

we examined expression levels in lavage fluid cells and lung tissue 18 h after inoculation. One lung was tied off at the bronchus, and a small piece of lung tissue from the middle lobe (an area adjacent to severe gross lesions in infected animals) was removed for RNA isolation. The other lung was lavaged for macrophage and neutrophil isolation. Northern blot analyses revealed elevated levels of TNF, IL-1, IL-6, and IL-8 mRNAs from *A. pleuropneumoniae*-inoculated animals compared with the levels in PBS/BSA-inoculated controls (Table 1). Interestingly, IL-6 mRNA was not detected by Northern analysis in lavage cells after infection, but IL-6 levels were significantly elevated in lung tissue from the same animals compared with PBS/BSA controls ($P < 0.005$; Table 1 and Fig. 3). Densitometric scanning of Northern results from four separate experiments showed that IL-1, IL-6, and IL-8 were present at low or undetectable levels in lavage cells and lung tissue in control animals, but were readily detected in inoculated animals (Table 1). In contrast, TNF was readily and consistently detected in lavage cells of all control animals at the same levels as in infected animals (Table 1). In lung tissue,

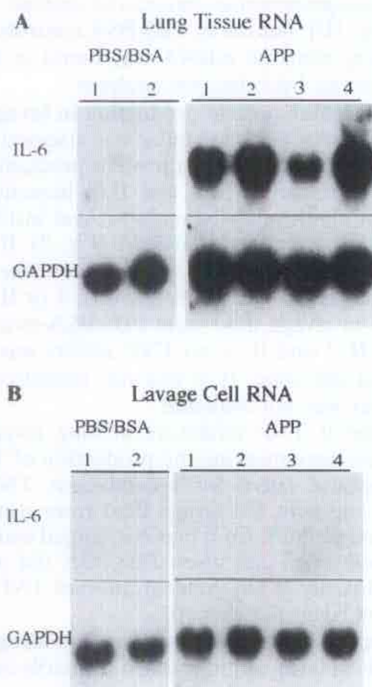

FIG. 3. IL-6 mRNA expression in lung tissue versus lavage cell total RNA. Lung (A) and lavage cell (B) total RNA samples from the same animals were collected approximately 18 h following either PBS/BSA or *A. pleuropneumoniae* (APP) inoculation. Membranes were hybridized sequentially for IL-6 and GAPDH as described in Materials and Methods.

however, TNF mRNA expression was significantly greater in *A. pleuropneumoniae*-inoculated pigs ($P = 0.04$).

Localization of cytokine expression to lesions. The relationship of inflammatory cytokine expression to focal lung lesions was investigated by in situ hybridization of lung tissue from animals 18 to 48 h after inoculation with *A. pleuropneumoniae* or PBS/BSA. Histopathologically, *A. pleuropneumoniae*-inoculated pigs produced lung lesions consistent with pleuropneumonia (Fig. 4A to E), while the lungs of sham-inoculated animals were unremarkable (Fig. 4F). Elevated levels of TNF, IL-1, and IL-8 mRNAs were present in lung tissue from animals inoculated with bacteria, especially at the sites of lesions (Fig. 4A to D). Inflammatory cells, including neutrophils and macrophages, were found within alveoli, interlobular spaces, and airways of *A. pleuropneumoniae*-inoculated animals. These areas also contained numerous cells staining positive for TNF, IL-1, and IL-8 (Fig. 4A to D). Coordinate expression of all three cytokines was always observed and was always associated with lung lesions. IL-6 expression was not examined in these experiments. Little or no TNF, IL-1, or IL-8 mRNA was detected in the lungs of uninfected controls (Fig. 4F for IL-1), and an IL-8 sense control probe did not hybridize to *A. pleuropneumoniae*-inoculated lung tissue (Fig. 4E). These results strongly suggest an association between inflammatory cytokine expression and *A. pleuropneumoniae* lung lesions.

Effect of heat-killed and low-virulence *A. pleuropneumoniae* on cytokine expression. It was possible that the elevated levels of inflammatory cytokines observed in the model were due to endotoxin or other common gram-negative bacterial products and not dependent on virulent *A. pleuropneumoniae*. To address this issue, two studies were performed to examine cytokine expression following inoculation with either heat-killed virulent *A. pleuropneumoniae* or a low-virulence *A. pleuropneumoniae* isolate.

In the first study, six pigs were divided into two groups of three and endotracheally inoculated with either 5×10^4 CFU of heat-killed *A. pleuropneumoniae* or virulent *A. pleuropneumoniae* organisms. One additional animal was included as a sham-infected control and received only PBS/BSA. After 24 h, all animals were killed, and lung lavage fluids were collected for RNA isolation and cytokine bioassay testing. *A. pleuropneumoniae* was reisolated from the lungs of all pigs inoculated with virulent *A. pleuropneumoniae*, whereas *A. pleuropneumoniae* was not reisolated from the lungs of pigs which received either heat-killed *A. pleuropneumoniae* or PBS/BSA. Severe lung lesions were present in the animals inoculated with virulent *A. pleuropneumoniae*, but no gross lesions were found in the lungs of animals inoculated with heat-killed *A. pleuropneumoniae* or PBS/BSA.

Lavage fluid cytokine profiles, as determined by Northern blotting and bioassay, did not differ significantly between animals which received heat-killed organisms and those which

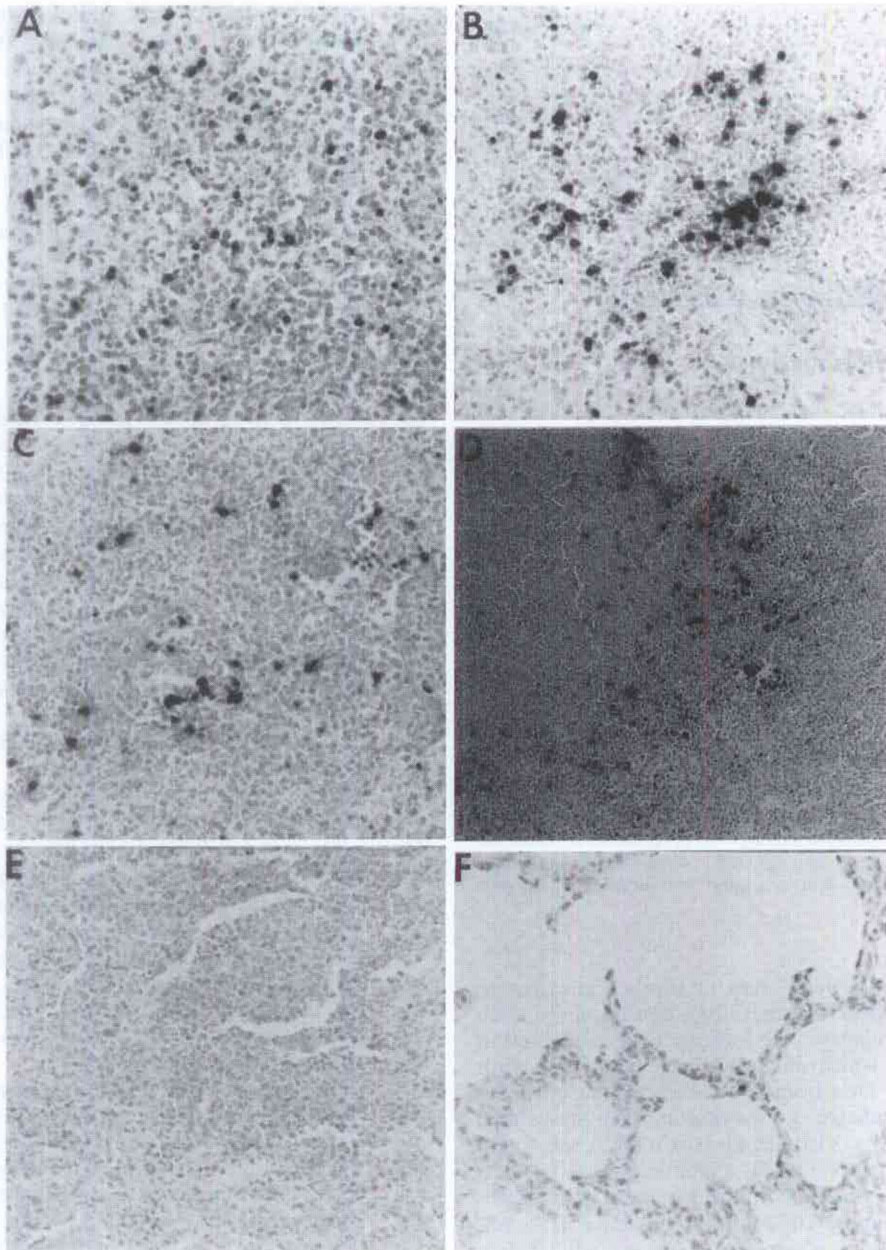

FIG. 4. Localization of TNF, IL-1, and IL-8 in lung lesions by in situ hybridization. Tissues were collected 18 to 48 h following *A. pleuropneumoniae* inoculation. Antisense oligonucleotide hybridizations were performed in lesions from infected animals for TNF (A), IL-1 (B and D), and IL-8 (C), and sense oligonucleotide hybridizations were performed for IL-8 (E). (F) Lung tissue from a PBS/BSA-inoculated animal hybridized with the IL-1 antisense probe. Magnification: (A, B, C, E, F) ×20; (D) ×10.

were inoculated with PBS/BSA. TNF mRNA was present at similar levels among all pigs, including those animals inoculated with virulent *A. pleuropneumoniae* (data not shown). IL-1 mRNA could only be detected in lavage cells from pigs inoculated with virulent *A. pleuropneumoniae*. These animals also had 20-fold-higher levels of bioactive IL-1 than pigs inoculated with heat-killed organisms or PBS/BSA. As in our kinetic studies, no IL-6 mRNA could be detected in lavage fluid cells from any of the animals, and no bioactive TNF could be detected in any of the lavage fluids.

Since serial in vitro passage of virulent *A. pleuropneumoniae* results in diminished pathogenic potential (1a, 28), a low-virulence *A. pleuropneumoniae* inoculum was prepared to determine if virulence was required for the rapid induction of cytokines early after infection.

Pigs were inoculated endotracheally with 5×10^4 CFU of low-virulence *A. pleuropneumoniae* or PBS/BSA as described above. As observed with the virulent *A. pleuropneumoniae* inoculum, vomiting also was observed within 30 min of inoculation with low-virulence *A. pleuropneumoniae*; otherwise, no abnormal signs were noted. At 2, 4, and 8 h following inoculation, the animals were killed, and lung lavage cells and fluid were collected for RNA isolation and bioassay. No gross lesions were present at necropsy, and *A. pleuropneumoniae* was not recultured from lungs.

Inflammatory cytokines could be detected in low-virulence

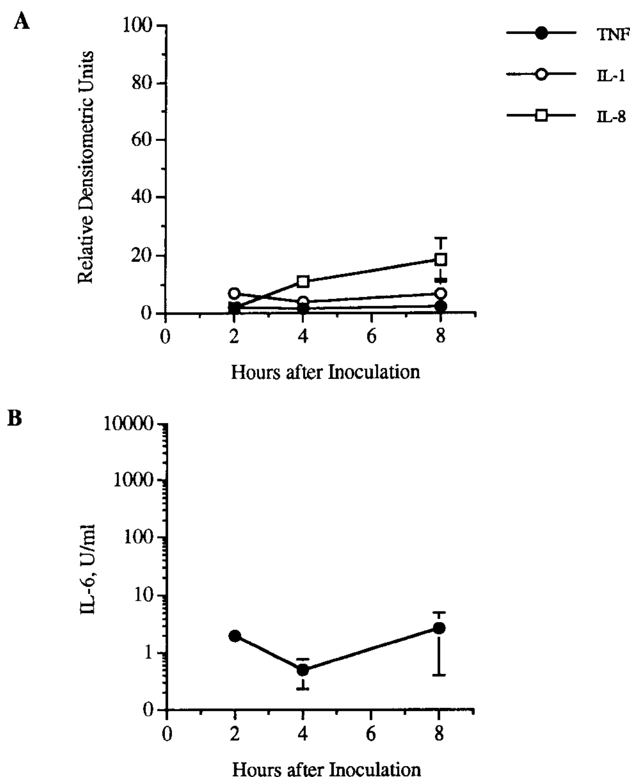

FIG. 5. Effect of inoculation with low-virulence *A. pleuropneumoniae* on cytokine expression in lung lavage cells and fluid. (A) Total RNA isolated from lavage cells was subjected to Northern blotting. The values shown represent the mean and SE for two pigs at 2 h or three pigs each at 4 and 8 h after inoculation. (B) Lavage fluids from the same animals as shown for Northern blotting were analyzed for IL-6 bioactivity.

A. pleuropneumoniae-inoculated pigs at levels equivalent to those in PBS/BSA controls. Northern blot densitometric analyses of total RNA from lavage cells indicated only low levels of TNF, IL-1, and IL-8, which did not change with time after inoculation (Fig. 5A). Densitometric values were similar for PBS/BSA- and low-virulence *A. pleuropneumoniae*-inoculated animals (data not shown). Virtually no IL-1 mRNA was found in this experiment, and therefore IL-1 bioassays were not performed. IL-6 bioactivity in lavage fluids from these animals was determined. Approximately 100-fold less bioactive IL-6 was found with low-virulence *A. pleuropneumoniae* as with virulent *A. pleuropneumoniae* (Fig. 5B versus Fig. 2). These data indicate that the induction of inflammatory cytokines requires an active infection and is not due only to the presence of endotoxin.

DISCUSSION

The pattern and kinetics of inflammatory cytokine expression in the pleuropneumonia model of acute respiratory disease are in general agreement with septic lung injury models (32, 37). We showed, by Northern blotting and in situ hybridization, that TNF, IL-1, IL-6, and IL-8 mRNA levels were elevated in the lung within hours of endotracheal instillation of *A. pleuropneumoniae* and that cytokine expression was localized to lesions. Lung tissue from uninfected control animals and from regions of *A. pleuropneumoniae*-inoculated animals which appeared normal histopathologically demonstrated very little or no TNF, IL-1, and IL-8 mRNA expression. These observations suggest that inflammatory cytokines may be involved in lesion development and/or progression during acute pleuropneumonia. IL-1 and IL-6 activity increased dramatically in lavage fluid after the inoculation of *A. pleuropneumoniae*, but unexpectedly, TNF expression was not induced in lavage cells or fluids after bacterial inoculation.

The principal observation appeared to be that IL-1 and IL-8 were expressed rapidly and to high levels in all sampling compartments. mRNA levels were elevated at the earliest time points after infection in both lung tissue and lavage cells. Twenty-five percent of lavage cells were positive for IL-1 and IL-8 by in situ hybridization at 2 h, whereas less than 10% of the cells were positive for IL-6 and TNF. Furthermore, IL-1 and IL-8 were specifically detected in lung tissue at the sites of lesions. We were unable to determine the cell types which were expressing cytokines. Swine cell surface markers for macrophages and neutrophils for use in formaldehyde-fixed tissues have not been described. Poor lavage cell morphology was a consistent observation following inoculation with virulent *A. pleuropneumoniae*. However, lavage cells obtained following inoculation with heat-killed *A. pleuropneumoniae* were easily identified by Wright stain. In the latter case, from 6 to 17% of the lavage cells were neutrophils, compared with 0 to 4% in samples from uninfected controls. The presence of heat-labile cytotoxin during acute pleuropneumonia is likely responsible for distorting cell morphology and further emphasizes the need for well-characterized antibodies specific for swine immune cells. In addition, staining morphology in fixed tissue or lavage cell smears could not be used because cellular appearance may be abnormal under disease conditions.

Rapid expression of IL-1 mRNA was correlated with the appearance and accumulation of IL-1 activity in lavage fluid. The approximately 1,000-fold increase in activity suggests that IL-1 may play a central role in the inflammatory response to *A. pleuropneumoniae* infection. It also suggests that IL-1 activity is not regulated in this model by production of IL-1 antagonist protein. We were unable to measure levels of IL-8 activity because a specific assay was not available. However, it is likely that IL-8 protein was present in lavage fluids from infected animals, since the IL-8 mRNA level was elevated in *A. pleuropneumoniae*-inoculated animals.

IL-6 mRNA was present in lung tissue homogenates, and high levels of IL-6 activity were present in lavage fluid, but IL-6 mRNA was only detected in lavage cells by in situ hybridization. The detection of IL-6 in lavage cells by in situ hybridization may be due to a greater sensitivity of in situ hybridization at the single-cell level. Nevertheless, the observations suggest that IL-6 expression is a major feature of pleuropneumonia and that the primary source is in lung tissue, perhaps fibroblasts or epithelial cells, rather than alveolar macrophages. In swine, LPS-activated alveolar macrophages appear to produce only low levels of IL-6, whereas fibroblasts treated with TNF or IL-1 express high levels of IL-6 (30a). Similarly, IL-6 mRNA was not detected in circulating leukocytes from patients with *Pseudomonas pseudomallei* sepsis, but plasma IL-6 bioactivity was readily detected (14).

TNF was observed in lung tissue specifically associated with lesions by in situ hybridization. However, it was the only cytokine routinely observed in lung tissue from control animals, and its mRNA levels were not elevated following infection. It was also expressed at low levels in lavage cells, although, as with IL-6, approximately 50% of lavage cells were positive for TNF mRNA at 4 to 24 h after infection. The failure to detect TNF activity in lavage fluids indicates that TNF protein is not produced or that inhibitors are present in lavage fluid. We were able to conclude that TNF inhibitors were not responsible for the lack of TNF bioactivity in our samples by evaluating the

inhibitory effects of lavage fluid on a recombinant TNF standard curve. Therefore, it appears that TNF mRNA is not actively translated during acute pleuropneumonia in the lung.

In contrast to TNF, biologically active IL-1 and IL-6 were detected at all time points after infection in lavage fluid from *A. pleuropneumoniae*-inoculated pigs. IL-6 may be a more reliable means to predict elevations in TNF and IL-1, since TNF and IL-1 induce IL-6 (11, 38). Furthermore, while soluble receptors or receptor antagonists for TNF and IL-1 interfere with their detection, soluble p80 receptors for IL-6 enhance its bioactivity (26). Levels of IL-6 have also been correlated with severity of disease, and IL-6 is often found concomitantly with IL-8 in models of sepsis (14, 16).

Heat-killed virulent organisms and a low-virulence isolate of *A. pleuropneumoniae* were used to exclude the possibility that the cytokine induction observed in these studies was due to endotoxin and did not require the establishment of an infection. The cytokine profiles observed following inoculation with heat-killed organisms and low-virulence *A. pleuropneumoniae* did not differ significantly from that observed following instillation of PBS/BSA. Since low-virulence *A. pleuropneumoniae* was not reisolated and no gross lesions were observed, it appeared that the organism was not capable of establishing an infection. Cytotoxin and hemolysin assays performed on the low-virulence organism indicated that it lacked activity compared with the parent strain (unpublished observation). These data indicate that the effects of virulent *A. pleuropneumoniae* cannot be explained by endotoxin alone and that other factors, perhaps related to colonization and toxin production, are required.

We were unable to detect IL-1 or IL-6 activity in serum samples from swine which had IL-1 and IL-6 in lavage fluid (data not shown). Thus, cytokine production appeared to be localized to the lung and not systemically disseminated. The ability to localize the release of cytokines may serve to limit the inflammatory response. The free exchange of cytokines across the blood-alveolar barrier is limited, even though both compartments contain cells capable of producing all of the inflammatory cytokines (25, 41). For example, inflammatory cytokines are frequently detected within the lungs of patients with adult respiratory distress syndrome, septicemia, and traumatic injury, while being difficult to detect in the blood (14, 35, 40).

These studies indicate that all of the inflammatory cytokines are expressed in response to pleuropneumonia, with TNF and IL-6 localized primarily to lung tissue, and IL-1 and IL-8 expressed both in tissue and in alveolar macrophages. IL-1 and IL-6, but not TNF, appear to be secreted and active in the alveoli. The expression of these cytokines is associated with the development of pleuropneumonia and may contribute to disease severity.

ACKNOWLEDGMENTS

We thank Evelyn Townsend for performing the IL-1 bioassays and Vicki King for assistance with statistical analysis.

This research was supported by the National Pork Producers Council, Minnesota Agriculture Experiment Station (MN 63-027), and United States Department of Agriculture National Research Initiative grant 93-37204-9338.

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