INFECTION AND IMMUNITY, Oct. 1995, p. 4084–4090 Vol. 63, No. 10
0019-9567/95/$04.00+0

Gene Expression and Production of Tumor Necrosis Factor Alpha, Interleukin 1, Interleukin 6, and Gamma Interferon in C3H/HeN and C57BL/6N Mice in Acute *Mycoplasma pulmonis* Disease

CHAD B. FAULKNER,[1]* JERRY W. SIMECKA,[2] MAUREEN K. DAVIDSON,[3] JERRY K. DAVIS,[3] TRENTON R. SCHOEB,[3] J. RUSSELL LINDSEY,[1,4] AND MICHAEL P. EVERSON[4,5]

Department of Comparative Medicine, The University of Alabama at Birmingham,[1] and The Veterans Administration Medical Center,[4] Birmingham, Alabama 35294; Department of Microbiology and Immunology, University of North Texas Health Science Center, Fort Worth, Texas 76107, and Mucosal Immunization Research Group, University of Alabama at Birmingham, Birmingham, Alabama 35294[2]; Division of Comparative Medicine, Department of Pathobiology, University of Florida, Gainesville, Florida 32610[3]; and Department of Medicine, Division of Clinical Immunology and Rheumatology, The University of Alabama at Birmingham, Birmingham, Alabama 35233[5]

Received 30 November 1994/Returned for modification 19 January 1995/Accepted 26 June 1995

Studies were conducted to determine whether the production of various cytokines is associated with *Mycoplasma pulmonis* disease expression. Susceptible C3H/HeN and resistant C57BL/6N mice were inoculated intranasally with 10^7 CFU of virulent *M. pulmonis* UAB CT or avirulent *M. pulmonis* UAB T. Expression of genes for tumor necrosis factor alpha (TNF-α), interleukin 1α (IL-1α), IL-1β, IL-6, and gamma interferon (IFN-γ) in whole lung tissue and TNF-α gene expression in bronchoalveolar lavage (BAL) cells was determined by reverse transcription-PCR using specific cytokine primers at various times postinoculation. In addition, concentrations of TNF-α, IL-1, IL-6, and IFN-γ were determined in BAL fluid and serum samples at various times postinoculation. Our results showed that there was a sequential appearance of cytokines in the lungs of infected mice: TNF-α, produced primarily by BAL cells, appeared first, followed by IL-1 and IL-6, which were followed by IFN-γ. Susceptible C3H/HeN mice had higher and more persistent concentrations of TNF-α and IL-6 in BAL fluid than did resistant C57BL/6N mice, indicating that TNF-α and possibly IL-6 are important factors in pathogenesis of acute *M. pulmonis* disease in mice. Serum concentrations of IL-6 were elevated in C3H/HeN mice, but not C57BL/6N mice, following infection with *M. pulmonis*, suggesting that IL-6 has both local and systemic effects in *M. pulmonis* disease.

Although acutely fatal pneumonia is not characteristic of mycoplasma infections in humans or animals, fatal cases of *Mycoplasma pneumoniae* infection do occur in humans and have been associated with the adult respiratory distress syndrome (ARDS) (19, 29). More recently, *Mycoplasma fermentans* infection has been associated with fulminant disease in patients with an initially flulike illness that rapidly progressed to ARDS or systemic disease with multiple organ system failure (35, 46). Susceptible mice experimentally infected with high doses of *Mycoplasma pulmonis* often die with an acute pneumonia characterized by neutrophilic exudate, alveolar edema, and pulmonary congestion and hemorrhage (10, 34). The nature of the lesions indicates that extensive lung vascular permeability changes occur in acute *M. pulmonis* disease. Except for pulmonary hemorrhage, similar lesions are seen in human patients who die of ARDS (45). One of the most common predisposing factors to the development of ARDS is gram-negative sepsis (25), and various cytokines have been implicated in the pathophysiology of gram-negative sepsis-associated lung injury (20, 51–54). Thus, lesions of acute *M. pulmonis* disease in mice may develop by similar mechanisms.

Murine respiratory mycoplasmosis (MRM), the natural disease caused by *M. pulmonis* in mice and rats, provides excellent rodent models for studies of the pathogenesis of both acute and chronic mycoplasmal respiratory disease (6, 7). There are differences in *M. pulmonis* disease expression among mouse strains, with C3H/HeN mice being far more susceptible than C57BL/6N mice, even though there is no difference in the number of mycoplasmas necessary to establish infection in these two mouse strains (13). Rather, the differences in susceptibility between C3H/HeN and C57BL/6N mice seem to be due to differences in the host response to *M. pulmonis* infection. The resistance of C57BL/6N mice is related to effective nonspecific pulmonary host defense mechanisms which limit the extent of infection (12, 13, 43). Intrapulmonary killing of *M. pulmonis* occurs within 4 h of experimental infection in C57BL/6N mice (12, 43). Paradoxically, a greater increase in inflammatory cells occurs in C3H/HeN mice, which have poor intrapulmonary killing of *M. pulmonis* (12, 43). By examining how these two mouse strains differ in their responses to *M. pulmonis* infection, it is likely that pathogenetic mechanisms influencing mycoplasmal respiratory disease expression can be identified. Using a mechanistic approach to disease expression should allow verification of pathogenic mechanisms involved in human mycoplasmal diseases.

Recent studies have shown that the genes of several cytokines are expressed in mice following experimental infection with either *M. pulmonis* (42) or *M. pneumoniae* (44), but little is known about the production of cytokines or their involvement in the pathogenesis of respiratory mycoplasmal infection. Cytokines are important mediators in both lung defense and inflammation (26). Resistance to a number of respiratory pathogens, including *Legionella pneumophila* (4), *Chlamydia trachomatis* (57, 58), and *Pneumocystis carinii* (8), is mediated

* Corresponding author. Present address: Division of Comparative Medicine, Department of Pathobiology, University of Florida, One Progress Blvd., P.O. Box 21, Alachua, FL 32615.

by various cytokines. In contrast, cytokine production also is involved in the pathogenesis of lung inflammatory diseases (27, 53, 54). For example, acute inflammatory lung injury due to gram-negative sepsis is known to involve a number of cytokines (5, 20, 41, 52). Intratracheal injection of lipopolysaccharide (LPS), a major cell wall component of gram-negative bacteria, causes an acute intra-alveolar neutrophilic exudate in rats. This response is associated with increased levels of tumor necrosis factor alpha (TNF-α) and interleukin 1 (IL-1) in the lung (53). Furthermore, another cytokine, IL-6, can modulate the effects of LPS-induced acute lung inflammation (54). Thus, further studies are needed to investigate the role of cytokines in the resistance and pathogenesis of respiratory disease in animals and humans.

The purpose of this study was to characterize both the gene expression and production of various cytokines and examine their association with the pathogenesis of acute *M. pulmonis* respiratory disease in mice. We chose to examine TNF-α, IL-1, IL-6, and gamma interferon (IFN-γ), since these cytokines have been found in association with inflammation (17, 31, 48) and with respiratory infections (4, 8, 57, 58). Furthermore, several mycoplasmal species have been found to induce various types of cells to produce TNF-α (2, 21, 32, 37, 47, 50), IFN-γ (28, 30), IL-1 (2, 28, 37), and IL-6 (37) in vitro. We characterized the expression and production of these cytokines in C3H/HeN and C57BL/6N mice after *M. pulmonis* infection and identified which of these cytokines was associated with the pathogenesis of acute mycoplasma respiratory disease. The results suggest that production of TNF-α and IL-6 has some effects on the differential expression of disease in these two mouse strains following *M. pulmonis* infection.

MATERIALS AND METHODS

Animals. C3H/HeN and C57BL/6N mice were obtained from the Frederick Cancer Research Facility, Frederick, Md., or from breeding colonies maintained at the University of Alabama at Birmingham (UAB). Animal colonies are monitored regularly by serology, culture, and histology for mycoplasmas and other murine pathogens (33). Serological testing for immunoglobulin M and immunoglobulin G antibodies to mycoplasmas (11) was done at UAB, and tests for antibodies to Sendai virus, pneumonia virus of mice, mouse hepatitis virus, minute virus of mice, mouse poliovirus, reovirus type 3, lymphocytic choriomeningitis virus, ectromelia, mouse adenovirus, epizootic diarrhea of infant mice, mouse pneumonitis virus, and polyomavirus were done by Charles River Professional Services, Wilmington, Mass. These colonies have been consistently free of all pathogens. Mice were reared in cages in Trexler plastic film isolators (Germ-Free Supply Division, Standard Safety Equipment Co., Palatine, Ill.) until weaning and then placed in sterilized microisolator cages (Lab Products, Maywood, N.J.). Sterile food (Agway, Syracuse, N.Y.) and water were provided ad libitum. All cages were provided with sterile hardwood chip bedding (P. J. Murphy Forest Products, Rochelle Park, N.J.), which was changed twice per week.

Animals were housed and maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* (40). Mice were anesthetized by intramuscular injection of ketamine HCl (8.7 mg/100 g of body weight; Aveco, Fort Dodge, Iowa) and xylazine (1.3 mg/100 g of body weight; Haver, Shawnee, Kans.) prior to inoculation or exsanguination.

Organisms. *M. pulmonis* UAB CT and UAB T were originally isolated from mice (10). All isolates were verified as pure cultures of *M. pulmonis* by immunofluorescence, and stock cultures of organisms were grown in broth A and stored at −70°C (10). The formulation for broth A is as follows (per liter): mycoplasma broth base (Frey's; Baltimore Biological Laboratories/BBL, Cockeysville, Md.), 22.5 g; DNA (degraded free acid from herring sperm; Sigma, St. Louis, Mo.), 0.2 g; ultrapure water, 785 ml; phenol red (Fisher Scientific Co., Springfield, N.J.), 2 ml; horse serum (gamma globulin free; GIBCO Diagnostics, Grand Island, N.Y.), 200 ml; 50% (wt/vol) glucose (Fisher), 10 ml; and cefoperazone sodium, 250-mg/ml stock solution (Roerig/Pfizer, New York, NY), 260 μl. Mice were inoculated intranasally with 10^7 CFU of virulent *M. pulmonis* UAB CT or avirulent *M. pulmonis* UAB T (10).

RNA isolation from lungs and bronchoalveolar lavage (BAL) cells. Total RNA was isolated from whole lungs of mice, using the Ultraspec RNA isolation system (Biotecx Laboratories, Inc., Houston, Tex.), which is based on the method described by Chomczynski and Sacchi (9). Briefly, lungs were homogenized in the Ultraspec RNA reagent. Chloroform was added to the homogenate and centrifuged at 12,000 × *g* (4°C) for 15 min. The RNA was precipitated by adding isopropanol to the aqueous phase and centrifuging the samples at 12,000 × *g* (4°C) for 10 min. The RNA pellet for each sample was washed twice with 75% ethanol by vortexing and subsequent centrifugation for 5 min at 7,500 × *g* and then resuspended in diethylpyrocarbonate-treated water. The concentration of RNA in each of the samples was determined spectrophotometrically (GeneQuant; Pharmacia, San Diego, Calif.), and samples were stored at −70°C until used.

The procedure used to isolate total RNA from the cellular fraction of BAL samples was similar to that used for RNA isolation from whole lungs, but because of the low number of cells obtained by lung lavage, we used RNATack resin (Biotecx Laboratories) when the RNA was precipitated with isopropanol. The resin binds the precipitated RNA, while the other impurities are removed by washing twice with 75% ethanol. The bound RNA was eluted from the resin by washing in diethylpyrocarbonate-treated water. The concentration of RNA in each of the samples was determined spectrophotometrically, and samples were stored at −70°C.

RT-PCR. The RNA was denatured by incubating it at 65°C for 2 min and then chilled quickly to 4°C. The reaction mixture for synthesis of cDNA by reverse transcription (RT) reaction included 25 mM $MgCl_2$; 10× reaction buffer containing 500 mM KCl, 100 mM Tris-HCl (pH 9.0), and 1.0% Triton X-100; 100 mM each dATP, dCTP, dGTP, and dTTP; recombinant RNase inhibitor (RNasin) (40 U/μl); and Moloney murine leukemia virus reverse transcriptase (200 U/μl) without RNase H activity. (All reagents were obtained from Promega, Madison, Wis.). The oligo$(dT)_{16}$ primer (50 μM) was synthesized at the UAB Oligonucleotide Core Facility. A 1-μl aliquot containing 50 ng of sample RNA was added to 9 μl of the RT master mix, which contained 5.6 mM $MgCl_2$, 1.1× of 10× reaction buffer (8.9 volumes of diethylpyrocarbonate-treated water added to 1.1 volumes of 10× reaction buffer), 1.1 mM each deoxynucleoside triphosphate, 1.1 U of RNasin, and 2.8 μM oligo$(dT)_{16}$ primer. The RT reaction was done in a thermocycler (PTC 100; MJ Research, Inc., Watertown, Mass.) at 42°C for 15 min, 37°C for 45 min, and 99°C for 5 min.

The PCR mixture contained 25 mM $MgCl_2$; 10× reaction buffer containing 500 mM KCl, 100 mM Tris-HCl (pH 9.0 at 25°C), and 1.0% Triton X-100; and *Taq* DNA polymerase (5 U/μl) (Promega). The primers for the cytokines and β-actin were synthesized at the UAB Oligonucleotide Core Facility. Primer sequences were as previously described (36, 39); the sequences of these primers (amplicon sizes are given in parentheses) were as follows: β-actin, 5′TGGAATC CTGTGGCATCCATGAAAC3′, 5′TAAAACGCAGCTCAGTAACAGTCCG3′ (348 bp); IL-1α, 5′CTCTAGAGCACCATGCTACA3′, 5′TGGAATCCAGGG GAAACACTG3′ (308 bp); IL-1β, 5′GCAACTGTTCCTGAACTCA3′, 5′CTCG GAGCCTGTAGTGCAG3′ (384 bp); IL-6, 5′TGGAGTCACAGAAGGAGTG GCTAAG3′, 5′TCTGACCACAGTGAGGAATGTCCAC3′ (155 bp); IFN-γ, 5′AGCGGCTGACTGAACTCAGATTGTAG3′, 5′GTCACAGTTTTCAGCTG TATAGGG3′ (249 bp); and TNF-α, 5′GGCAGGTCTACTTTGGAGTC3′, 5′ACATTCGAGGCTCCAGTGAATTCGG3′ (307 bp).

For each reaction, 40 μl of master mix containing 1.25 mM $MgCl_2$, 10× reaction buffer, 0.03 U of *Taq* DNA polymerase, and 15 μM each primer was added to a tube containing 10 μl of the cDNA synthesized in the RT reaction. The tubes were incubated in a thermocycler (MJ Research) at 95°C for 2 min (once), 94°C for 45 s, 60°C for 2 min, and 72°C for 3 min (20 to 35 cycles), and 72°C for 7 min, and then held at 4°C. PCR products were visualized by ethidium bromide staining after agarose (2%) gel electrophoresis.

To determine whether *M. pulmonis* infection induced expression of cytokine mRNAs, we determined the number of PCR amplification cycles resulting in little or no PCR product for each cytokine in total RNA from lung and BAL cells from sham-inoculated control mice, and we amplified samples from inoculated mice accordingly. The numbers of cycles were 25 for TNF-α, 25 for IL-1α, 21 for IL-1β, 30 for IL-6, 30 for IFN-γ, and 30 for β-actin, unless stated otherwise. PCR amplifications without RT ensured that RNA preparations were not contaminated with genomic DNA. We attempted to obtain semiquantitative assessment of cytokine gene expression by comparison of PCR product band intensity with that of β-actin mRNA amplified for 30 PCR cycles.

BAL. BAL fluid samples were collected as previously described (12). Briefly, a sterile 19-gauge intravenous catheter (Deseret Medical, Becton-Dickinson, Inc., Sandy, Utah) was inserted into the tracheas of mice, and 1.0-ml washes were done three times with sterile, endotoxin-free, 0.9% NaCl without preservatives (Abbott Laboratories, North Chicago, Ill.). Lavage samples were separated into cellular and noncellular fractions by centrifugation at 400 × *g* for 10 min at 4°C. To remove mycoplasmas, noncellular fractions were filter sterilized with 0.1-μm-pore-size low-protein-binding syringe filters (Gelman Sciences, Ann Arbor, Mich.) and stored at −70°C.

Cytokine immunoassays. Concentrations of TNF-α in serum and BAL fluid samples were determined by using an enzyme-linked immunosorbent assay (ELISA) for murine TNF-α (Genzyme Corp., Cambridge, Mass.). IL-6 and IFN-γ concentrations were determined by using the appropriate paired antibodies and recombinant murine cytokines in an ELISA format (PharMingen, San Diego, Calif.) according to manufacturer's instructions. In brief, each assay was a solid-phase enzyme immunoassay that used a monoclonal antibody to capture the appropriate cytokine. Goat polyclonal antibody against TNF-α was used for amplification in the TNF-α assay, and peroxidase-conjugated donkey anti-goat

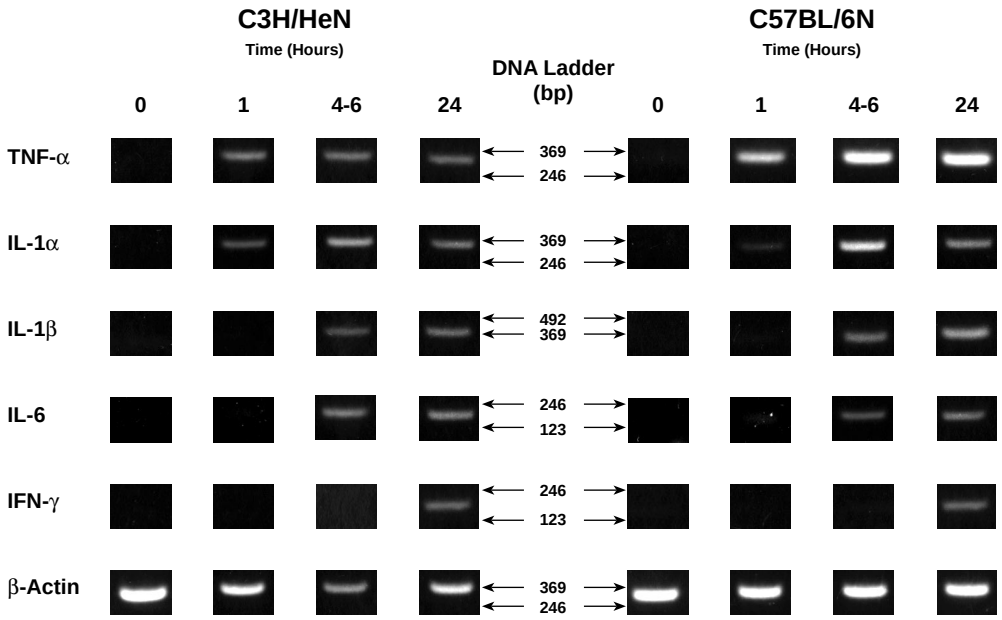

FIG. 1. Cytokine gene expression in the lungs of C3H/HeN and C57BL/6N mice inoculated with *M. pulmonis* UAB CT. Cytokine mRNAs were detected by RT-PCR of 50 ng of total lung RNA. The experiments were done twice with four mice of each strain at each time p.i. Levels of gene expression for the indicated cytokines for one representative animal for each experiment are shown.

antibody was used for detection. For IL-6 and IFN-γ assays, a biotinylated anticytokine monoclonal antibody was used for detection and developed with an avidin-peroxidase conjugate. For each run, a reference standard curve was generated with different concentrations of recombinant murine TNF-α, IL-6, or IFN-γ.

IL-1 bioassay. The term IL-1 refers to two distinct polypeptide gene products, IL-1α and IL-1β, which recognize the same cell surface receptors and have overlapping biologic activities (15). Because there were no commercially available immunoassays that distinguish mouse IL-1α and IL-1β, we used a biologic assay to determine the presence or absence of IL-1 in serum and BAL fluid samples. This bioassay measures the ability of IL-1 to stimulate the proliferation of the murine T-cell cloned line D-10M (24). Briefly, duplicate wells of serially diluted serum and BAL fluid samples were incubated with 10^4 D-10M cells per well in RPMI 1640 medium containing 2 mM L-glutamine, 20 μg of gentamicin per ml, and 50 μM 2-mercaptoethanol at 37°C in the presence of 8% CO_2. After 68 h, each well was pulsed with 1 μCi of [^{3}H]thymidine and harvested 4 h later onto glass fiber filters, which were dried and counted in a liquid scintillation counter. For each run, IL-1 concentrations in samples were determined by extrapolation from a reference curve generated with different concentrations of murine IL-1α.

Statistical analysis. Data were analyzed by analysis of variance followed by Tukey's multigroup comparison for parametric data or Mann-Whitney *U* tests using Bonferroni-adjusted probabilities for nonparametric data (56). Analyses were performed by using the SYSTAT computer program (SYSTAT, Inc., Evanston, Ill.). A *P* value of less than 0.05 was considered significant.

RESULTS

Cytokine gene expression in lungs of mice inoculated with *M. pulmonis* UAB CT. In each of two experiments, four mice of each strain were studied at 1, 4 to 6, and 24 h postinoculation (p.i.), and four sham-inoculated control mice of each strain were included. Although expression of β-actin mRNA was uniform among control mice of the same strain, expression appeared to be slightly higher in C57BL/6N mice than in C3H/HeN mice, and expression was inconsistent in mice of both strains after inoculation with *M. pulmonis*. Consequently, these results are best interpreted conservatively as qualitative rather than semiquantitative, precluding an assessment of relative levels of expression of cytokine genes between susceptible C3H/HeN mice and resistant C57BL/6N mice. Nonetheless, a consistent pattern in the order of appearance of cytokine mRNAs was evident in the first 24 h after *M. pulmonis* UAB CT infection in the lungs of mice of both strains (Fig. 1); mRNAs of TNF-α and IL-1α were detected as early as 1 h p.i., and those of IL-1β and IL-6 were detected by 4 to 6 h p.i., whereas IFN-γ mRNA was not evident until 24 h p.i. (Table 1).

TNF-α gene expression in lung lavage cells from mice infected with *M. pulmonis* UAB CT. Alveolar macrophages are strong producers of TNF-α (16, 49); to evaluate their role in production of TNF-α in lungs of *M. pulmonis* infected mice, we assessed mRNA TNF-α expression in whole lung and BAL cells from C3H/HeN and C57BL/6N mice inoculated with virulent *M. pulmonis*. Because of differences in expression of β-actin mRNA between C57BL/6N and C3H/HeN mice and among inoculated mice of both strains, these results must be interpreted as qualitative rather than semiquantitative. However, with 23 amplification cycles, TNF-α mRNA was readily detectable in BAL cells, but not lungs, at 4 and 24 h p.i. (Fig. 2), indicating that BAL cells are a major source of the TNF-α protein detected in BAL fluid samples.

TNF-α response to *M. pulmonis* infection. Initially, seven C3H/HeN mice were inoculated intranasally with *M. pulmonis*

TABLE 1. Expression of cytokine genes in lungs of mice after intranasal inoculation of 10^7 CFU *M. pulmonis* UAB CT

Cytokine mRNA[a]	Expression			
	Control	At indicated time after *M. pulmonis* infection		
		1 h	4–6 h	24 h
TNF-α	−	+	+	+
IL-1α	−	+	+	+
IL-1β	−	−	+	+
IL-6	−	−	+	+
IFN-γ	−	−	−	+
β-Actin	+	+	+	+

[a] The presence of cytokine mRNAs was detected by RT-PCR of 50 ng of total lung RNA.

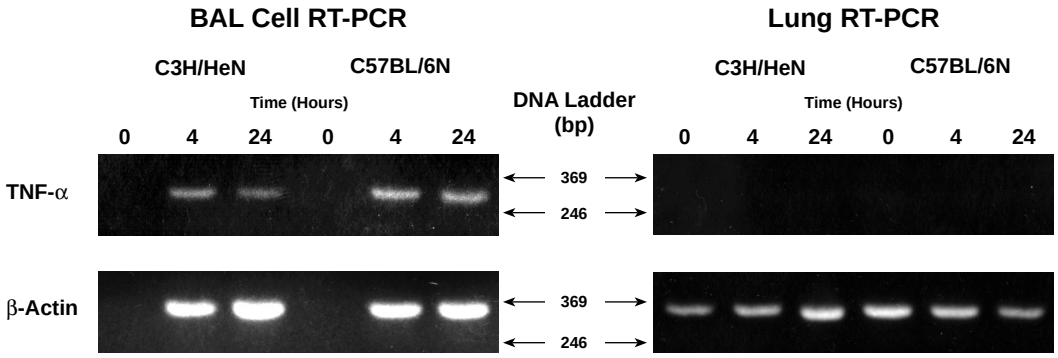

FIG. 2. TNF-α gene expression in the lungs of C3H/HeN and C57BL/6N mice inoculated with *M. pulmonis* UAB CT. TNF-α mRNA was detected by RT-PCR of 50 ng of total RNA from either BAL cells or whole lung.

UAB CT, and seven control mice were sham inoculated with sterile broth. The mice were killed 4 days p.i., and serum and BAL fluid were collected. BAL fluids of control mice did not contain detectable TNF-α, whereas those of infected mice had 259 ± 193 pg of TNF-α per ml ($P \leq 0.001$). TNF-α was not detected in the serum samples of either control or infected mice.

BAL fluid TNF-α responses were further studied in C3H/HeN and C57BL/6N mice inoculated either with virulent UAB CT or avirulent UAB T strains of *M. pulmonis*. Two to eight infected mice were sacrificed for collection of BAL fluid and serum 0.5, 1, 2, 4, and 24 h and 3 and 7 days p.i. At least one control mouse of each strain was sacrificed at each time point. Lungs from at least two mice for each combination of mouse and mycoplasma strain and each p.i. interval were examined histologically; all had lesions typical of acute MRM (10, 34). Results for *M. pulmonis* UAB CT are shown in Fig. 3, and those for strain UAB T are shown in Fig. 4. Significant increases in TNF-α occurred in mice of both strains after infection with *M. pulmonis* UAB CT; however, concentrations were higher, and remained elevated longer, in C3H/HeN mice than in C57BL/6N mice. Infection with the avirulent UAB T strain of *M. pulmonis* also resulted in significantly increased TNF-α in BAL fluid at early times p.i., but the increases did not persist as long as in mice given the virulent strain of *M. pulmonis*. TNF-α was not detected in BAL fluids of the control mice or in sera of either infected or control mice.

IL-1 and IFN-γ response to *M. pulmonis* infection. Serum and BAL fluid IL-1 responses were compared between C3H/HeN and C57BL/6N mice inoculated with the UAB CT strain of *M. pulmonis* at 4 and 24 h after infection (five mice per group). Control mice were inoculated with sterile broth. Mice of both strains had significantly increased IL-1 in BAL fluid after inoculation, but there were no statistically significant differences between mice of the two strains (Fig. 5). IL-1 was not detected in the sera of control or infected mice or in the BAL fluids of control mice.

To determine whether IFN-γ is produced in infected mice, sera and BAL fluids were collected from C3H/HeN and C57BL/6N mice inoculated with *M. pulmonis* UAB CT at 0.5, 1, 2, 4, and 24 h and at 3 and 7 days p.i. (at least four mice per group). Sham-inoculated control mice of each strain were included. In two experiments, IFN-γ was undetectable in either serum or BAL fluid (data not shown).

IL-6 response to *M. pulmonis* infection. Serum and BAL fluid IL-6 responses in C3H/HeN and C57BL/6N mice were compared by immunoassay at 0.5, 1, 2, 4, and 24 h and at 3 and 7 days after infection with *M. pulmonis* UAB CT. Each group included sham-inoculated control mice and three to nine inoculated mice. IL-6 concentrations in BAL fluid were significantly increased in C3H/HeN by 4 h p.i., were maximally increased at 3 days p.i., and remained elevated for at least 7 days p.i. (Fig. 6). In contrast, BAL fluid concentrations of IL-6 were significantly increased in C57BL/6N mice only at 24 h p.i. BAL fluid concentrations of IL-6 were significantly higher in C3H/HeN mice than in C57BL/6N mice at 3 and 7 days p.i. Serum concentrations of IL-6 were significantly increased in infected C3H/HeN mice at 3 days p.i. (Fig. 7) but not increased in infected C57BL/6N mice (data not shown).

DISCUSSION

The fibrinous and hemorrhagic pneumonia observed in mice that die within 1 week of inoculation with high doses of virulent *M. pulmonis* (10, 34) indicates that extensive lung vascular permeability changes occur in acute MRM. Similar lesions are seen in human patients who die of ARDS, except that pulmonary hemorrhage is uncommon (45). One of the most common predisposing factors to the development of ARDS is gram-negative sepsis (25). Cytokines have been implicated as important mediators in the pathophysiology of gram-negative sepsis-associated lung injury (20, 51–54). The purpose of the present study was to investigate the expression and production of several cytokines in the lungs of mice following infection with *M.*

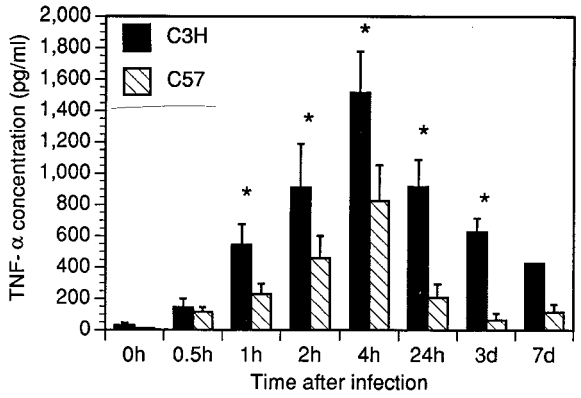

FIG. 3. TNF-α concentrations in BAL fluids of mice inoculated with virulent *M. pulmonis* UAB CT. Bars represent mean (± standard error) TNF-α concentrations, with $n = 5$ to 15 mice for each mean. There were significant differences ($P < 0.05$) between the means for C3H/HeN and C57BL/6N mice at the indicated times p.i. (indicated by asterisks).

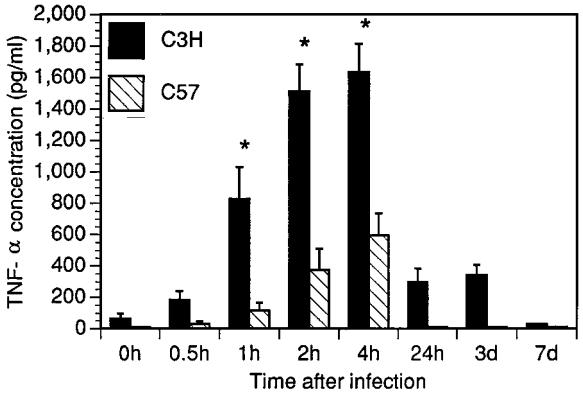

FIG. 4. TNF-α concentrations in BAL fluids of mice inoculated with avirulent *M. pulmonis* UAB T. Bars represent mean (± standard error) TNF-α concentrations, with $n = 5$ to 15 mice for each mean. There were significant differences ($P < 0.05$) between the means for C3H/HeN and C57BL/6N mice at the indicated times p.i. (indicated by asterisks).

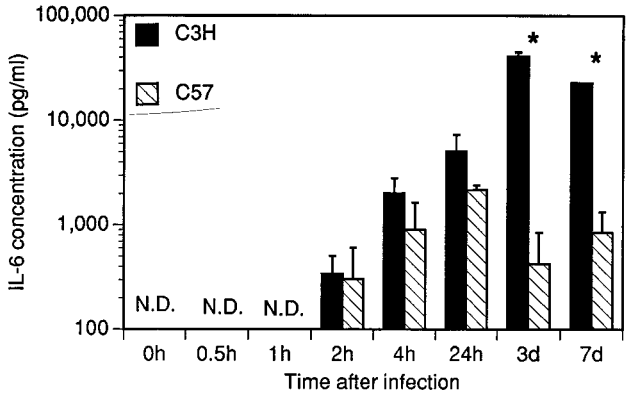

FIG. 6. IL-6 concentrations in BAL fluids from mice inoculated with *M. pulmonis* UAB CT. Bars represent mean (± standard deviation) IL-6 concentrations, with $n = 3$ to 9 for each mean. IL-6 concentrations were significantly increased ($P < 0.05$) in C3H/HeN mice by 4 h p.i. and remained elevated for at least 7 days (d) p.i. compared with sham-inoculated (0 h) control C3H/HeN mice. In contrast, IL-6 concentrations were significantly increased ($P < 0.05$) only at 24 h p.i. in C57BL/6N mice. There were significant differences ($P < 0.05$) between the means of C3H/HeN and C57BL/6N mice at 3 and 7 days p.i. (indicated by asterisks. N.D., not detectable.

pulmonis. Although differences in expression of β-actin mRNA between C57BL/6N and C3H/HeN mice and among inoculated mice of both strains precluded assessment of relative levels of cytokine gene expression, the results nonetheless showed a consistent sequential activation of cytokine production after infection with *M. pulmonis* in both susceptible and resistant mice. TNF-α and IL-1α mRNAs were present as early as 1 h after *M. pulmonis* infection, and TNF-α protein appeared in BAL fluid coincident with the appearance of TNF-α mRNA. IL-1β and IL-6 mRNAs also appeared rapidly, being evident by 4 h p.i., whereas IFN-γ mRNA appeared after 6 h p.i. and was evident 24 h p.i. Coincident with their mRNAs, IL-1 and IL-6 were detected in BAL fluid at 4 h PI. However, IFN-γ was not detected in BAL fluid at any time after infection, suggesting that IFN-γ was not produced, was present at very low concentrations, and/or was rapidly utilized.

We were somewhat surprised that no β-actin PCR product was obtained with BAL cell samples from sham-inoculated control mice (Fig. 2). We also were unable to demonstrate any PCR product for the gene encoding glyceraldehyde-3-phosphate dehydrogenase, another commonly used housekeeping gene, with these samples. This might indicate that BAL cells are transcriptionally inert and require a stimulus to express genes that are constitutively expressed in most other cell types.

Our results differ somewhat from those of a previous study in which not only IFN-γ mRNA but also that of TNF-α did not increase until 24 h after *M. pulmonis* infection (42). However, our results are consistent with those of other studies showing a sequential appearance of TNF-α, IL-1, and IL-6 in serum after intravenous injection of a lethal dose of *Escherichia coli* in baboons (20, 52). By using this model of sepsis, TNF-α was also shown to be important in subsequent in vivo production of IL-1 and IL-6 (20). In addition, TNF-α production by macrophages is known to stimulate the production of IFN-γ by natural killer cells (3). Thus, our studies are consistent with the idea that TNF-α is important for the progressive appearance of inflammation-associated cytokines following *M. pulmonis* infection.

The results of this study implicate TNF-α production in the pathogenesis of MRM. TNF-α concentrations in BAL fluid were higher and more persistent in susceptible C3H/HeN mice than in resistant C57BL/6N mice after infection with the virulent UAB CT strain of *M. pulmonis*. Infection with the aviru-

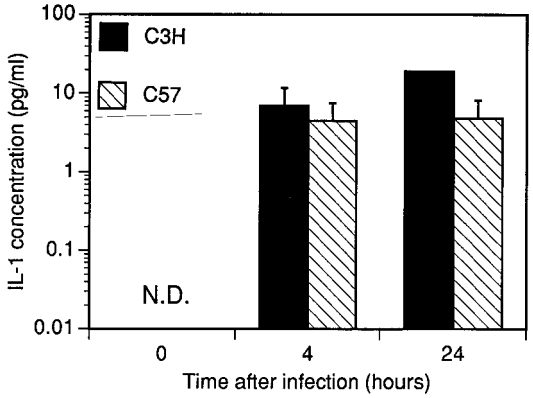

FIG. 5. IL-1 concentrations in BAL fluids from mice inoculated with *M. pulmonis* UAB CT. Bars represent mean (± standard deviation) IL-1 concentrations in pooled BAL fluid samples (equal volumes from all animals), with $n = 5$ mice per group for each of two experiments. There were no significant differences between C3H/HeN and C57BL/6N mice at any time. N.D., not detectable.

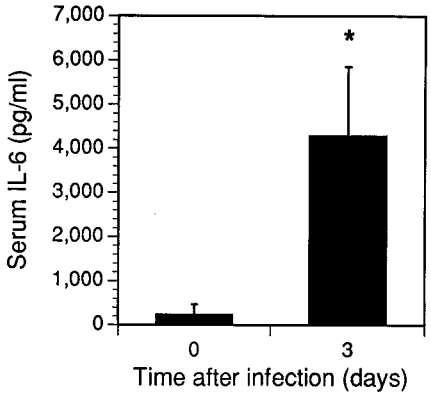

FIG. 7. IL-6 concentrations in sera from C3H/HeN mice inoculated with *M. pulmonis* UAB CT. Bars represent mean (± standard deviation) IL-6 concentrations, with $n = 3$ to 7 for each mean. Means of sham-inoculated control mice and infected mice were significantly different ($P < 0.05$) at 3 days p.i.

lent UAB T strain of *M. pulmonis* resulted in much lower TNF-α concentrations, which returned to control levels by 24 h p.i. TNF-α also is an important mediator of neutrophil recruitment to sites of inflammation, including inflammatory responses within the lung (53), and is directly chemotactic for neutrophils and increases their adherence to vascular endothelium (22). This finding is consistent with the observation that C3H/HeN mice accumulate much larger numbers of neutrophils in their lungs in response to *M. pulmonis* than do C57BL/6N mice (43). However, TNF-α alone is probably not solely responsible for the acute inflammatory lesions of MRM. IL-1, which also is produced after infection with *M. pulmonis*, is another potent mediator of neutrophil recruitment (53) and may act synergistically with TNF-α.

TNF-α, along with other cytokines, has a role in antimicrobial host defense mechanisms (3, 14, 38). However, its role in host defense in MRM is presently uncertain. Susceptible C3H/HeN mice, in which intrapulmonary killing of *M. pulmonis* is less efficient than in resistant C57BL/6N mice, produce higher concentrations of TNF-α in their lungs, although alveolar macrophages appear to be the major effector cells of intrapulmonary killing of *M. pulmonis* in C57BL/6N mice (12). Our results and those of Nishomoto et al. (42) suggest that alveolar macrophages are activated following *M. pulmonis* infection and are a major source of TNF-α in both mouse strains. The concentrations of IL-1, another macrophage-produced cytokine, in lung lavages were not different between the two mouse strains, whereas the TNF-α response to *M. pulmonis* infection differed greatly. Thus, alveolar macrophages differ between the two mouse strains in production of TNF-α, but in *M. pulmonis* disease, this cytokine may be associated more with severity of pulmonary injury than with host defense against the mycoplasma.

In addition to TNF-α, our results show that IL-6 production also is associated with *M. pulmonis* disease progression, inasmuch as *M. pulmonis* infection results in much higher BAL fluid concentrations of IL-6 in susceptible C3H/HeN mice than in resistant C57BL/6N mice. This difference may be due to the higher concentrations of TNF-α in the lungs of infected C3H/HeN mice, because TNF-α can induce IL-6 gene expression and protein production (1). IL-6 was maximally elevated in the lungs of infected C3H/HeN mice by 3 days p.i., which temporally coincides with influx of the greatest numbers of neutrophils following *M. pulmonis* infection (43). One possible role for IL-6 in the pathogenesis of MRM is the modulation of the inflammatory response to *M. pulmonis* infection. This notion is supported by the observation that IL-6 reduces the numbers of neutrophils in acute lung inflammation following the intratracheal inoculation of LPS in rats (54). Conversely, the presence of circulating IL-6 in individuals with meningococcal septic shock connotes a poor outcome (55). A similar association of serum IL-6 and severe disease was found in our studies, inasmuch as serum IL-6 concentrations increased after *M. pulmonis* inoculation only in susceptible C3H/HeN mice.

The increased circulating concentrations of IL-6 in infected C3H/HeN mice is consistent with our previous studies (18) that suggest the development of an acute-phase response in mice with severe *M. pulmonis* disease, inasmuch as IL-6 is the most potent inducer of the acute phase response (23). Presently, the role of IL-6 in the pathogenesis of MRM is unclear. However, our results suggest that IL-6 may have both local and systemic effects during disease progression.

In summary, our results showed a sequential appearance of TNF-α, followed by IL-1 and IL-6, in the lungs of mice inoculated with *M. pulmonis*. Susceptible C3H/HeN mice had higher and more persistent amounts of TNF-α in BAL fluid than did resistant C57BL/6N mice in response to either the virulent or avirulent strain of *M. pulmonis*, and this difference was more pronounced in mice given the virulent strain. There also were greater amounts of IL-6 in BAL fluid in C3H/HeN mice, and there was increased serum IL-6 in C3H/HeN mice but not C57BL/6N mice. Thus, differences in production of TNF-α and IL-6 could be factors in the difference in susceptibility of C3H/HeN and C57BL/6N mice to *M. pulmonis.*

ACKNOWLEDGMENTS

We thank J. Erwin, D. McDuffie, and P. Patel for excellent technical assistance; S. Cartner and K. Beagley for helpful discussions; and G. H. Cassell for support.

This work was supported by Public Health Service grants RR00463, RR07003, and AI15128 from the National Institutes of Health and by funds from the Veterans Administration Research Service.

REFERENCES

1. **Akira, S., T. Hirano, T. Taga, and T. Kishimoto.** 1990. Biology of multifunctional cytokines: IL-6 and related molecules (IL-1 and TNF). FASEB J. **4:**2860–2867.
2. **Almeida, R. A., M. J. Wannemuehler, and R. F. Rosenbusch.** 1992. Interaction of *Mycoplasma dispar* with bovine alveolar macrophages. Infect. Immun. **60:**2914–2919.
3. **Bancroft, G. J., K. C. F. Sheehan, R. D. Schreiber, and E. R. Unanue.** 1989. Tumor necrosis factor is involved in the T cell-independent pathway of macrophage activation in scid mice. J. Immunol. **143:**127–130.
4. **Blanchard, D. K., J. Y. Djeu, T. W. Klein, H. Friedman, and W. E. Stewart II.** 1988. Protective effects of tumor necrosis factor in experimental *Legionella pneumophila* infections of mice via activation of PMN function. J. Leukocyte Biol. **43:**429–435.
5. **Brigham, K. L., and B. Meyrick.** 1986. Endotoxin and lung injury. Am. Rev. Respir. Dis. **133:**913–927.
6. **Cassell, G. H.** 1982. Derrick Edward Award Lecture. The pathogenic potential of mycoplasmas: *Mycoplasma pulmonis* as a model. Rev. Infect. Dis. **4**(Suppl)**:**S18–S34.
7. **Cassell, G. H., J. K. Davis, J. W. Simecka, J. R. Lindsey, N. R. Cox, S. E. Ross, and M. Fallon.** 1986. Mycoplasmal infections: disease pathogenesis, implications, for biomedical research, and control, p. 87–130. *In* P. N. Bhatt, R. O. Jacoby, H. C. Morse, and A. E. New (ed.), Viral and mycoplasmal infections of laboratory rodents: effects on biomedical research. Academic Press, Orlando, Fla.
8. **Chen, W., E. A. Havell, L. L. Moldawer, K. E. McIntyre, R. A. Chizzonite, and A. G. Harmsen.** 1992. Interleukin-1: an important mediator of host resistance against *Pneumocystis carinii*. J. Exp. Med. **176:**713–718.
9. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
10. **Davidson, M. K., J. R. Lindsey, R. F. Parker, J. G. Tully, and G. H. Cassell.** 1988. Differences in virulence for mice among strains of *Mycoplasma pulmonis*. Infect. Immun. **56:**2156–2162.
11. **Davis, J. K., G. H. Cassell, G. Gambill, N. R. Cox, H. W. Watson, and M. K. Davidson.** 1987. Diagnosis of murine mycoplasmal infections by enzyme-linked immunosorbent assay (ELISA). Isr. J. Med. Sci. **23:**717–722.
12. **Davis, J. K., M. K. Davidson, T. R. Schoeb, and J. R. Lindsey.** 1992. Decreased intrapulmonary killing of *Mycoplasma pulmonis* after short-term exposure to NO_2 is associated with damaged alveolar macrophages. Am. Rev. Respir. Dis. **145:**406–411.
13. **Davis, J. K., R. F. Parker, H. White, D. Dziedzic, G. Taylor, M. K. Davidson, N. R. Cox, and G. H. Cassell.** 1985. Strain differences in susceptibility to murine respiratory mycoplasmosis in C57BL/6 and C3H/HeN mice. Infect. Immun. **50:**647–654.
14. **Denis, M.** 1991. Tumor necrosis factor and granulocyte macrophage-colony stimulating factor stimulate human macrophages to restrict growth of virulent *Mycobacterium avium* and to kill avirulent *M. avium*: killing effector mechanism depends on the generation of reactive nitrogen intermediates. J. Leukocyte Biol. **49:**380–387.
15. **Dinarello, C. A.** 1991. Interleukin-1 and interleukin-1 antagonism. Blood **77:**1627–1652.
16. **Everson, M. P., and D. B. Chandler.** 1992. Changes in distribution, morphology, and tumor necrosis factor-alpha secretion of alveolar macrophage subpopulations during the development of bleomycin-induced pulmonary fibrosis. Am. J. Pathol. **140:**503–512.
17. **Farrar, M. A., and R. D. Schreiber.** 1993. The molecular cell biology of interferon-γ and its receptor. Annu. Rev. Immunol. **11:**571–611.
18. **Faulkner, C. B., M. K. Davidson, J. K. Davis, T. R. Schoeb, J. W. Simecka, and J. R. Lindsey.** 1994. Intravascular coagulation in acute *Mycoplasma pulmonis* infection in mice. Lab. Anim. Sci. **45:**368–372.

19. **Fischman, R. A., K. E. Marschall, J. W. Kislak, and D. M. Greenbaum.** 1978. Adult respiratory distress syndrome caused by *Mycoplasma pneumoniae*. Chest **74:**471–473.
20. **Fong, Y., K. J. Tracey, L. L. Moldawer, D. G. Hesse, K. B. Manogue, J. S. Kenney, A. T. Lee, G. C. Kuo, A. C. Allison, S. F. Lowry, et al.** 1989. Antibodies to cachectin/TNF reduce interleukin-1-β and interleukin-6 appearance during lethal bacteremia. J. Exp. Med. **170:**1627–1633.
21. **Gallily, R., T. Sher, P. Ben-Av, and J. Loewenstein.** 1989. Tumor necrosis factor as a mediator of *Mycoplasma orale*-induced tumor cell lysis by macrophages. Cell. Immunol. **121:**146–153.
22. **Gamble, J. R., J. M. Harlan, S. J. Klebanoff, and M. A. Vadas.** 1985. Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. Proc. Natl. Acad. Sci. USA **82:**8667–8671.
23. **Gauldie, J., C. Richards, D. Harnish, P. Lansdorp, and H. Baumann.** 1987. Interferon beta 2/B-cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. Proc. Natl. Acad. Sci. USA **84:**7251–7255.
24. **Hopkins, S. J., and M. Humphreys.** 1989. Simple, sensitive and specific bioassay of interleukin-1. J. Immunol. Methods **120:**271–276.
25. **Hyers, T. M., and A. A. Fowler.** 1986. Adult respiratory distress syndrome: causes, morbidity, and mortality. Fed. Proc. **45:**25–29.
26. **Kelley, J.** 1990. Cytokines of the lung. Am. Rev. Respir. Dis. **141:**765–788.
27. **Kern, J. A., R. J. Lamb, J. C. Reed, J. A. Elias, and R. P. Daniele.** 1988. Interleukin-1-beta gene expression in human monocytes and alveolar macrophages from normal subjects and patients with sarcoidosis. Am. Rev. Respir. Dis. **137:**1180–1184.
28. **Kirchner, H., A. Bauer, T. Moritz, and F. Herbst.** 1986. Lymphocyte activation and induction of interferon gamma in human leucocyte cultures by the mitogen in *Mycoplasma arthritidis* supernatant (MAS). Scand. J. Immunol. **24:**609–613.
29. **Koletsky, R. J., and A. J. Weinstein.** 1980. Fulminant *Mycoplasma pneumoniae* infection. Report of a fatal case and a review of the literature. Am. Rev. Respir. Dis. **122:**491–496.
30. **Lai, W. C., M. Bennett, S. P. Pakes, V. Kumar, D. Steutermann, I. Owusu, and A. Mikhael.** 1990. Resistance to *Mycoplasma pulmonis* mediated by activated natural killer cells. J. Infect. Dis. **161:**1269–1275.
31. **Le, J., and J. Vilcek.** 1987. Biology of disease. Tumor necrosis factor and interleukin-1: cytokines with multiple overlapping physiologic activities. Lab. Invest. **56:**234–248.
32. **Lin, Y., J. L. Collins, P. G. Case, and P. Q. Patek.** 1988. Effect of mycoplasmas on natural cytotoxic activity and release of tumor necrosis factor alpha by spleen cells. Infect. Immun. **56:**3072–3075.
33. **Lindsey, J. R.** 1986. Prevalence of viral and mycoplasmal infections in laboratory rodents, p. 801–808. *In* P. N. Bhatt, R. O. Jacoby, H. C. Morse, and A. E. New (ed.), Viral and mycoplasmal infections of laboratory rodents: effects on biomedical research. Academic Press, Orlando, Fla.
34. **Lindsey, J. R., and G. H. Cassell.** 1973. Experimental *Mycoplasma pulmonis* infection in pathogen-free mice. Models for studying mycoplasmas of the respiratory tract. Am. J. Pathol. **72:**63–90.
35. **Lo, S.-C., M. S. Dawson, P. B. Newton III, M. A. Sonoda, J. W.-K. Shih, W. F. Engler, R. Y.-H. Wang, and D. J. Wear.** 1989. Association of the virus-like infectious agent originally reported in patients with AIDS with acute fatal disease in previously healthy non-AIDS patients. Am. J. Trop. Med. Hyg. **41:**364–376.
36. **Montgomery, R. A., and M. J. Dallman.** 1991. Analysis of cytokine gene expression during fetal thymic ontogeny using the polymerase chain reaction. J. Immunol. **147:**554–560.
37. **Muhlradt, P. F., and U. Schade.** 1991. MDHM, a macrophage-stimulatory product of *Mycoplasma fermentans*, leads to in vitro interleukin-1 (IL-1), IL-6, tumor necrosis factor, and prostaglandin production and is pyrogenic for rabbits. Infect. Immun. **59:**3969–3974.
38. **Murray, H. W.** 1990. Gamma interferon, cytokine-induced macrophage activation, and antimicrobial host defense in vitro, in animal models, and in humans. Diagn. Microbiol. Infect. Dis. **13:**413–421.
39. **Murray, L. J., R. Lee, and C. Martens.** 1990. In vivo cytokine gene expression in T cell subsets of the autoimmune MRL/Mp-*lpr*/*lpr* mouse. Eur. J. Immunol. **20:**163–170.
40. **National Research Council.** 1985. A guide for the care and use of laboratory animals. A report of the Institute of Laboratory Animal Resources Committee on care and use of laboratory animals. National Institutes of Health publication no. 85-23. U.S. Department of Health and Human Services, Washington, D.C.
41. **Niederman, M. S., and A. M. Fein.** 1990. Sepsis syndrome, the adult respiratory distress syndrome, and nosocomial pneumonia. A common clinical sequence. Clin. Chest Med. **11:**633–656.
42. **Nishimoto, M., A. Akashi, K. Kuwano, C. Tseng, K. Ohizumi, and S. Arai.** 1994. Gene expression of tumor necrosis factor α and interferon γ in the lungs of *Mycoplasma pulmonis*-infected mice. Microbiol. Immunol. **38:**345–352.
43. **Parker, R. F., J. K. Davis, D. K. Blalock, R. B. Thorp, J. W. Simecka, and G. H. Cassell.** 1987. Pulmonary clearance of *Mycoplasma pulmonis* in C57BL/6N and C3H/HeN mice. Infect. Immun. **55:**2631–2635.
44. **Pietsch, K., S. Ehlers, and E. Jacobs.** 1994. Cytokine gene expression in the lungs of BALB/c mice during primary and secondary intranasal infection with *Mycoplasma pneumoniae*. Microbiology **140:**2043–2048.
45. **Pratt, P. C.** 1982. Pathology of adult respiratory distress syndrome: implications regarding therapy. Semin. Respir. Med. **4:**79–85.
46. **Saillard, C., P. Carle, J. M. Bove, C. Bebear, S. C. Lo, J. W. Shih, R. Y. Wang, D. L. Rose, and J. G. Tully.** 1990. Genetic and serologic relatedness between *Mycoplasma fermentans* strains and a mycoplasma recently identified in tissues of AIDS and non-AIDS patients. Res. Virol. **141:**385–395.
47. **Sher, T., S. Rottem, and R. Gallily.** 1990. *Mycoplasma capricolum* membranes induce tumor necrosis factor α by a mechanism different from that of lipopolysaccharide. Cancer Immunol. Immunother. **30:**86–92.
48. **Sherry, B., and A. Cerami.** 1988. Cachectin/tumor necrosis factor exerts endocrine, paracrine, and autocrine control of inflammatory responses. J. Cell Biol. **107:**1269–1277.
49. **Spatafora, M., A. Merendino, M. Gjomarkaj, G. Chiappara, M. Melis, V. Bellia, and G. Bonsignore.** 1988. Tumor necrosis factor release by pulmonary alveolar macrophages. Respiration **54**(Suppl. 1)**:**73–77.
50. **Sugama, K., K. Kuwano, M. Furukawa, Y. Himano, T. Satoh, and S. Arai.** 1990. Mycoplasmas induce transcription and production of tumor necrosis factor in a monocytic cell line, THP-1, by a protein kinase C-independent pathway. Infect. Immun. **58:**3564–3567.
51. **Tracey, K. J., B. Beutler, S. F. Lowry, J. Merryweather, S. Wolpe, I. W. Milsark, R. J. Hariri, T. J. Fahey, A. Zentella, J. D. Albert, G. T. Shires, and A. Cerami.** 1986. Shock and tissue injury induced by recombinant human cachectin. Science **234:**470–474.
52. **Tracey, K. J., Y. Fong, D. G. Hesse, K. R. Manogue, A. T. Lee, G. C. Kuo, S. F. Lowry, and A. Cerami.** 1987. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteremia. Nature (London) **330:**662–664.
53. **Ulich, T. R., L. R. Watson, S. Yin, K. Guo, P. Wang, H. Thang, and J. del Castillo.** 1991. The intratracheal administration of endotoxin and cytokines. I. Characterization of LPS-induced IL-1 and TNF mRNA expression and the LPS-, IL-1, and TNF-induced inflammatory infiltrate. Am. J. Pathol. **138:** 1485–1496.
54. **Ulich, T. R., S. Yin, K. Guo, E. S. Yi, D. Remick, and J. del Castillo.** 1991. Intracheal administration of endotoxin and cytokines. II. Interleukin-6 and transforming growth factor beta inhibit acute inflammation. Am. J. Pathol. **138:**1097–1101.
55. **Waage, A., P. Brandtzaeg, A. Halstensen, P. Kierulf, and T. Espevik.** 1989. The complex pattern of cytokines in serum from patients with meningococcal septic shock. J. Exp. Med. **169:**333–338.
56. **Wilkinson, L.** 1989. SYSTAT: the system for statistics. SYSTAT, Inc., Evanston, Ill.
57. **Williams, D. M., G. I. Byrne, B. Grubbs, T. J. Marshal, and J. Schachter.** 1988. Role in vivo for gamma interferon in control of pneumonia caused by *Chlamydia trachomatis* in mice. Infect. Immun. **56:**3004–3006.
58. **Williams, D. M., D. M. Magee, L. F. Bonewald, J. G. Smith, C. A. Bleicker, G. I. Byrne, and J. Schachter.** 1990. A role in vivo for tumor necrosis alpha in host defense against *Chlamydia trachomatis*. Infect. Immun. **58:**1572–1576.