Pulmonary Immunity to *Pseudomonas aeruginosa* in Intestinally Immunized Rats: Roles of Alveolar Macrophages, Tumor Necrosis Factor Alpha, and Interleukin-1α

A. BURET,[1,2,3]* M. L. DUNKLEY,[2,3] G. PANG,[2,3] R. L. CLANCY,[1,2,3] AND A. W. CRIPPS[1,2,3]

Australian Institute of Mucosal Immunology,[1] and Department of Pathology, Faculty of Medicine, University of Newcastle, Royal Newcastle Hospital,[2] Newcastle, New South Wales 2300, and Hunter Area Pathology Service, Division of Molecular and Cell Biology, John Hunter Hospital, New Lambton Heights, New South Wales 2305,[3] Australia

The aims of this study were to assess the role played by alveolar macrophages, tumor necrosis factor alpha (TNF-α), and interleukin-1α (IL-1α) in pulmonary immunity against *Pseudomonas aeruginosa* in animals that have been immunized via the gut-associated lymphoid tissue. Following intra-Peyer's patch immunization and subsequent intratracheal challenge with live bacteria, significantly enhanced bacterial clearance from the lungs correlated with an increase in bronchoalveolar neutrophils, increased recruitment and phagocytic activity of alveolar macrophages, and accelerated production of TNF-α in the bronchoalveolar space, while levels of IL-1α remained low. Administration of recombinant TNF-α in physiological concentrations did not affect the proliferation of *P. aeruginosa* in vitro, but when given intratracheally to rats at the time of infection, recombinant TNF-α significantly increased bacterial clearance from the lungs. In these animals, phagocytic activity of bronchoalveolar neutrophils was enhanced, while the recruitment of alveolar macrophages and neutrophils remained unchanged. In acutely infected nonimmune animals, bronchoalveolar concentrations of soluble IL-1α and TNF-α increased until the time of death. Levels of prostaglandin E_2 and thromboxane B_2 were similar in each experimental group. These results indicate that infection in immune animals enhanced both recruitment and phagocytic activity of alveolar macrophages as well as induced an accelerated production of TNF-α. In immune challenged animals, this cytokine enhanced the phagocytic activity of neutrophils and improved bacterial clearance from the lung. Levels of soluble IL-1α and TNF-α in nonimmune rats increased consistently following infection until the time of death, thus implicating these cytokines in the pathogenesis of acute *P. aeruginosa* pneumonia.

Infection with *Pseudomonas aeruginosa* is the major cause of morbidity and mortality in patients with cystic fibrosis (14). A number of studies have attempted to develop a vaccine against *P. aeruginosa* (reviewed in reference 3), but protective immunity and prevention of colonization in the lung have not been achieved. Moreover, the effector mechanisms of immunity against *P. aeruginosa* remain poorly understood. Our laboratory has developed an animal model in which intestinal immunization of rats with killed *P. aeruginosa* protects against subsequent pulmonary infection and significantly enhances survival (7). In this model, an enhancement of both neutrophil recruitment and activity was shown to be a central effector mechanism of protective immunity following intestinal immunization (5). Thus, cellular events appear to play a critical role in host defense against *P. aeruginosa*, an observation which is consistent with the findings of other studies (18, 20, 25, 27, 36). However, the mediators responsible for the activation of neutrophils in the immune lung remained undefined. In the airways, alveolar macrophages are pivotal effector cells of protective immunity because of their phagocytic activity, their role as an antigen-presenting cell, and their release of numerous products such as proteases, oxidizing agents, complement components, eicosanoids, and cytokines (1, 15, 27). Interleu-

kin-1 (IL-1) and tumor necrosis factor (TNF), two proinflammatory cytokines mainly produced by macrophages, exist as two distinct polypeptides (IL-1α and IL-1β and TNF-α and TNF-β) (9). IL-1α and TNF-α share a wide range of biological activities, including the activation of neutrophils (9, 21, 29, 30, 33). In addition, it has been suggested that TNF-α on its own is lethal to *Pneumocystis carinii* (24) and capable of binding to gram-negative bacteria and altering their virulence properties (17). Other reports have shown that intramuscular or intraperitoneal administration of recombinant IL-1α or recombinant IL-1β, respectively, protected mice from lethal systemic *P. aeruginosa* infections (22, 37). In keeping with our efforts to further define the effector mechanisms of pulmonary immunity against *P. aeruginosa*, the aim of this study was to assess whether protective immunity in the lung following intestinal immunization was associated with an enhanced recruitment and/or activity of alveolar macrophages, which in turn may increase neutrophil activation via an increased production of TNF-α and/or IL-1α. The results indicate that (i) following a challenge infection, recruitment and early phagocytic activity of alveolar macrophages are significantly increased in animals that had been previously immunized, (ii) bronchoalveolar production of TNF-α but not IL-1α is accelerated in these rats, (iii) TNF-α on its own does not inhibit proliferation of *P. aeruginosa* in vitro, and (iv) intratracheal inoculation of recombinant TNF-α (rTNF-α) at the time of infection improves bacterial clearance by enhancing the phagocytic activity of bronchoalveolar neutrophils.

* Corresponding author. Present address: Gastrointestinal Research Group, University of Calgary, Pediatrics, Health Sciences Centre, Room 1764, 3330 Hospital Dr. N.W., Calgary, Alberta, Canada T2N 4N1.

MATERIALS AND METHODS

Animal model. Male, specific-pathogen-free DA rats, 8 to 10 weeks old and weighing 180 to 280 g (mean [$\bar{x}$] ± standard error of the mean [SEM], 222 ± 3 g), were used throughout this study as described previously (5, 7). Animals were fed commercial rodent pellets, given water ad libitum, and maintained under microisolator hoods in a specific-pathogen-free environment with 12-h-light–12-h-dark photoperiods until the start of the experimental protocol. Maintenance of animals and experimental procedures were carried out in accordance with the guidelines of the Ethics Committee of the University of Newcastle (Newcastle, New South Wales, Australia).

Bacteria. A mucoid *P. aeruginosa* isolate, serotype II, phage type 21/44/109/119X/1214 (Central Public Health Laboratory, London, United Kingdom), originally isolated from a patient with cystic fibrosis, was used and prepared in all experiments as described previously (5, 7). Briefly, *P. aeruginosa* was grown on chocolate agar plates overnight at 37°C, harvested, suspended, and washed in sterile phosphate-buffered saline (pH 7.3; 0.15 M NaCl; PBS), quantitated, and checked for purity by being replated for single-colony growth on chocolate agar. For immunization, bacteria were killed by exposure to 1% paraformaldehyde for 2 h and washed three times in sterile PBS.

Immunization procedures. Rats were immunized intra-Peyer's patch as described previously (5, 7). Other studies have established that intra-Peyer's-patch immunization mimics intestinal immunization and specifically induces gut-derived immune mechanisms, unlike intraperitoneal immunization which primes both mucosal and systemic immunity (11). Killed *P. aeruginosa* cells were suspended in sterile PBS to a concentration of 10^{10} CFU/ml as determined by photometry (A_{405}) and emulsified with an equal volume of incomplete Freund's adjuvant. Following intravenous anesthesia of the rats with 3.6% chloral hydrate, 1 to 2 μl of the preparation was injected subserosally into each Peyer's patch of the rats (designated immune animals). A second group of sham-treated animals (nonimmune animals) was inoculated with incomplete Freund's adjuvant-PBS only. A third group of animals (control rats) was not immunized.

Challenge and bacterial clearance. Fourteen days postimmunization, nonimmune and immune rats were challenged intratracheally via a catheter (20 gauge by 2 in. [ca. 5 cm]) with 50 μl of PBS containing 5×10^8 CFU of live autologous *P. aeruginosa* harvested from an overnight culture on chocolate agar. Control animals received PBS only. Rats were killed with an intraperitoneal overdose of pentabarbitone sodium (Nembutal; Boehringer Ingelheim, Artarmon, New South Wales, Australia) at 30 min, 4 h, 12 h, 24 h, and 7 days after intratracheal inoculation. After challenge, blood samples were collected by cardiac puncture, and bronchial washings were obtained by five sequential lavages with 2 ml of sterile PBS. Serum aliquots were frozen and kept at −70°C. Viable *P. aeruginosa* bacteria recovered in the bronchoalveolar lavage fluids (BAL) were counted by serial dilution and culture on nutrient agar for 18 h at 37°C. The remainder of each lavage sample was centrifuged for 10 min at 250 × g and 5°C. Aliquots from the supernatant were frozen and maintained at −70°C until further study. Bacterial recovery was expressed as $\log_{10}$ CFU, and bacterial clearance in the immune animals was expressed as the percentage of cleared bacteria compared with the number of microorganisms recovered from nonimmune rats. Previous studies (2, 7) of pulmonary infections with *P. aeruginosa* or other microorganisms have clearly demonstrated that bacterial recovery from BAL was a reliable parameter which correlates with total bacterial clearance from the lungs.

Alveolar macrophage counts and phagocytic indices. Total bronchoalveolar cell counts were determined with a hemocytometer. In addition, 100 μl of each fresh BAL was spun for 10 min at 20 × g onto a microscope slide with a cytospin (Shandon, Inc., Pittsburgh, Pa.), and cytocentrifuged specimens were fixed and stained in Diff Quick (Veterinary Medical and Surgical Supply Pty. Ltd., Maryville, Australia) to allow the calculation of percentages of alveolar macrophages. Total numbers of alveolar macrophages were obtained by multiplying these percentages by the total number of bronchoalveolar cells. Total numbers of neutrophils were counted in a similar fashion. Cytospin slides were washed to remove free extracellular bacteria not associated with phagocytes, and the preparations were further evaluated under light microscopy for the determination of two phagocytic indices, i.e., (i) the percentage of alveolar macrophages that had phagocytosed 1 to 10 bacteria and (ii) the percentage of alveolar macrophages that phagocytosed more than 10 bacteria, as described previously (5).

Isolation and purification of alveolar macrophages. For the isolation of alveolar macrophages, BAL were centrifuged for 10 min at 250 × g and 5°C. The pellet was then resuspended in 1 ml of sterile PBS containing 0.1% (wt/vol) bovine serum albumin (BSA; Sigma-Aldrich, Bioscientific Ltd., Sydney, Australia). The cells in PBS-BSA were layered over a gradient of 20 and 60% Percoll (Pharmacia, Uppsala, Sweden) and centrifuged for 30 min at 2,000 × g and 10°C. Alveolar macrophages were harvested from the 20 to 60% interface, washed in sterile PBS-BSA, recentrifuged at 250 × g for 10 min at 10°C, and suspended in 1 ml of sterile PBS. Cells were evaluated for viability by exclusion of trypan blue (0.1%), and the preparation was assessed microscopically for purity with a cytospin preparation as described above. Throughout the study, alveolar macrophage populations obtained in this fashion were >99% pure and >90% viable.

Electron microscopy. Phagocytosis of bacteria by alveolar macrophages was verified by use of transmission electron microscopy. Cells purified from immune animals after 30 min of bacterial challenge were centrifuged for 10 min at 250 × g and 5°C, fixed overnight at 5°C in Karnovsky's fixative (2.5% glutaraldehyde, 2% formaldehyde), postfixed for 2 h in 1% osmium tetroxide, dehydrated in ethanol, and embedded in Spurr's medium (FSE, Sydney, Australia). Sections (80 nm) were stained with saturated uranyl acetate in 50% ethanol and 0.04% lead citrate. Micrographs were obtained with a Philips CM10 transmission electron microscope at an acceleration voltage of 80 kV.

TNF-α and IL-1α assays. Levels of soluble IL-1α and TNF-α were measured in nondiluted BAL samples and diluted (1:2) serum specimens. The TNF-α concentration was determined with a mouse TNF-α enzyme-linked immunosorbent assay (ELISA) kit as described in the manufacturer's instructions (Genzyme, Cambridge, Mass.). It has been previously established that murine TNF-α and rat TNF-α are cross-reactive and that this mouse ELISA kit could be used to detect rat TNF-α (6). Briefly, this ELISA is a solid-phase enzyme immunoassay employing the multiple-antibody sandwich principle in which TNF-α present in the samples is captured by a hamster monoclonal antibody for murine TNF-α previously adsorbed onto the wells. Following subsequent incubations with a goat polyclonal anti-murine TNF antibody, and with a horseradish peroxidase-conjugated donkey anti-goat immunoglobulin, the peroxidase enzyme reacts with the peroxide substrate and the chromogen *o*-phenylenediamine dihydrochloride added to the wells. The intensity of the color reaction, which is proportional to the amount of TNF-α from the sample, was read at A_{492}.

TABLE 1. Bacterial recoveries (CFU) and total alveolar macrophage and neutrophil counts in BAL from nonimmune
and immune rats challenged for 0.5 and 4 h with live *P. aeruginosa*

Challenge time and rat group	Bacteria		Alveolar macrophages		Neutrophils	
	Log$_{10}$ CFU (% clearance)[a]	P	Count (10^5)[a]	P	Count (10^5)[a]	P
0.5 h						
Nonimmune (n = 6–19)	7.63 ± 0.05		3.66 ± 0.44		0.059 ± 0.02	
Immune (n = 6–18)	7.11 ± 0.10* (69.8)	<0.0001	4.44 ± 0.50	>0.26	0.261 ± 0.04*	<0.01
4 h						
Nonimmune (n = 4–12)	7.73 ± 0.08		1.81 ± 0.27		10.77 ± 1.99	
Immune (n = 6–13)	6.07 ± 0.09* (97.8)	<0.0001	23.78 ± 8.16*	<0.04	109.8 ± 27.03*	<0.01

[a] Values are $\bar{x}$s ± SEMs. *, P < 0.05, nonimmune compared with immune values.

The assay was set up for a detection limit of 50 pg of TNF-α per ml. This ELISA kit has no detectable cross-reactivity with other cytokines (IL-1α, IL-2, IL-3, granulocyte-macrophage colony-stimulating factor and gamma interferon) (Genzyme). Soluble IL-1α was measured with a competitive radioimmunoassay test kit which specifically quantitates rat IL-1α (Cytokine Sciences, Inc., Boston, Mass.). After incubating the primary rabbit anti-rat IL-1α antibody in the presence of IL-1 from the samples, ^{125}I-radiolabelled recombinant rat IL-1α is added to the tubes as a tracer. Both the primary antibody and the tracer are used in limiting quantities. Hence, the amount of tracer that can bind to the primary antibody is inversely proportional to the amount of free IL-1 in the sample. A secondary antibody, sheep anti-rabbit immunoglobulin G, is then used to immunoprecipitate IL-1α–primary antibody complexes. Bound tracer is separated from unbound tracer by centrifugation, and the immunoprecipitate is counted directly with a gamma scintillation counter. The detection limit for this assay was 100 pg/ml of sample.

Effect of TNF-α on bacterial proliferation in vitro. In vitro dose response curves of bacterial proliferation versus physiological concentrations of TNF-α (covering the range of the levels measured in the in vivo experiments) were determined. Live *P. aeruginosa* bacteria (5 × 10^8) in 100 μl of PBS were added to 5 ml of nutrient broth (0.86% peptone, 0.64% NaCl in dH$_2$O [pH 7.5]) prewarmed at 37°C. Increasing concentrations (0 [PBS alone], 0.28, 0.83, 2.5, 5, and 10 ng) of recombinant murine TNF-α (Genzyme) were added immediately to the preparations. Following incubation for 4 h at 37°C in a shaking incubator (200 rpm), viable bacteria in each tube were quantitated by serial dilution and culture on nutrient agar for 18 h at 37°C as described above. Bacterial proliferation factors were obtained from the ratio of CFU in each preparation at 4 h to the initial CFU at the time of inoculation.

Effects of TNF-α in vivo. Another set of experiments assessed the effects in vivo of rTNF-α on bacterial proliferation, neutrophil and macrophage recruitment, and phagocytic indices. Physiological concentrations (1, 4, and 10 ng) of murine rTNF-α (Genzyme), which is cross-reactive with rat TNF-α (6), were added to the intratracheal bolus of 5 × 10^8 live *P. aeruginosa* bacteria as described above and given to nonimmune animals. The results were compared with those obtained from one group of nonimmune rats that received *P. aeruginosa* in PBS only. Animals were killed 3 h postchallenge, and the BAL were collected for assessment of bacterial recovery, macrophage and neutrophil counts, and calculation of macrophage and neutrophil phagocytic indices, as described above.

PGE$_2$ and TXB$_2$ assays. Prostaglandin E$_2$ (PGE$_2$) and thromboxane B$_2$ (TXB$_2$) synthesis was measured in nondiluted and diluted sera and BAL samples (serum, 1:200; BAL, 1:10)

with a competitive enzyme immunometric assay kit as described in the instructions provided by the manufacturer (Cayman Chemical Company, Sapphire Bioscience, Alexandria, New South Wales, Australia). Briefly, the immunoassay for each eicosanoid was based on the competition between free PGE$_2$ or TXB$_2$ in the samples and acetylcholinesterase-linked PGE$_2$ or TXB$_2$ tracer for limited specific rabbit antiserum-binding sites. Thus, the amount of tracer that is able to bind the rabbit antiserum will be inversely proportional to the concentration of free eicosanoid. The eicosanoid-antiserum complexes bind to a mouse monoclonal anti-rabbit antibody previously adsorbed onto the well, and after the addition of Ellman's reagent, a color reaction which can be measured spectrophotometrically (A_{414}) develops. The amount of free PGE$_2$ or TXB$_2$ is inversely proportional to the intensity of the color reaction. The PGE$_2$ immunoassay is 100% specific for PGE$_2$, 9.2% for 15-keto PGE$_2$, 5% for PGE$_1$, and <0.2% for TXB$_2$ and other prostaglandins. The TXB$_2$ immunoassay is 100% specific for TXB$_2$, 8.2% for 2,3-dinor TXB$_2$, 0.2% for 11-dehydro-TXB$_2$, and <0.01% for PGE$_2$, PGF$_{1α}$, and leukotriene B$_4$ (LTB$_4$).

Statistical analysis. All results were expressed as $\bar{x}$ ± SEM and compared by one-way analysis of variance and then by Tukey's test for multiple-comparison analysis when applicable. When results were expressed as percentages, all values underwent an arcsine transformation prior to statistical comparison and were transformed back to percentages for the expression of $\bar{x}$ ± SEM. Levels of P of <0.05 were considered significant.

RESULTS

Bacterial clearance and macrophage counts. Bacterial recoveries and numbers of macrophages and neutrophils counted in the BAL are given in Table 1. Intra-Peyer's-patch immunization with killed bacteria resulted in significantly enhanced bacterial clearance from the lung after subsequent pulmonary challenge with live *P. aeruginosa*. Compared with nonimmunized rats, immune animals had cleared 69.8% bacteria from their lungs after 30 min and 97.8% after 4 h. This rate of clearance was associated with a significantly enhanced recruitment of alveolar macrophages 4 h after challenge, while neutrophil recruitment was significantly increased at 30 min and 4 h. The numbers of alveolar macrophages in immune and nonimmune animals were not different prior to challenge (data not shown).

Phagocytic indices. Examination of cytospin preparations under light microscopy allowed easy identification of phagocytes that had ingested *P. aeruginosa* (Fig. 1). Intracellular localization of the phagocytosed bacteria was confirmed with transmission electron microscopy (Fig. 1). After 30 min of live
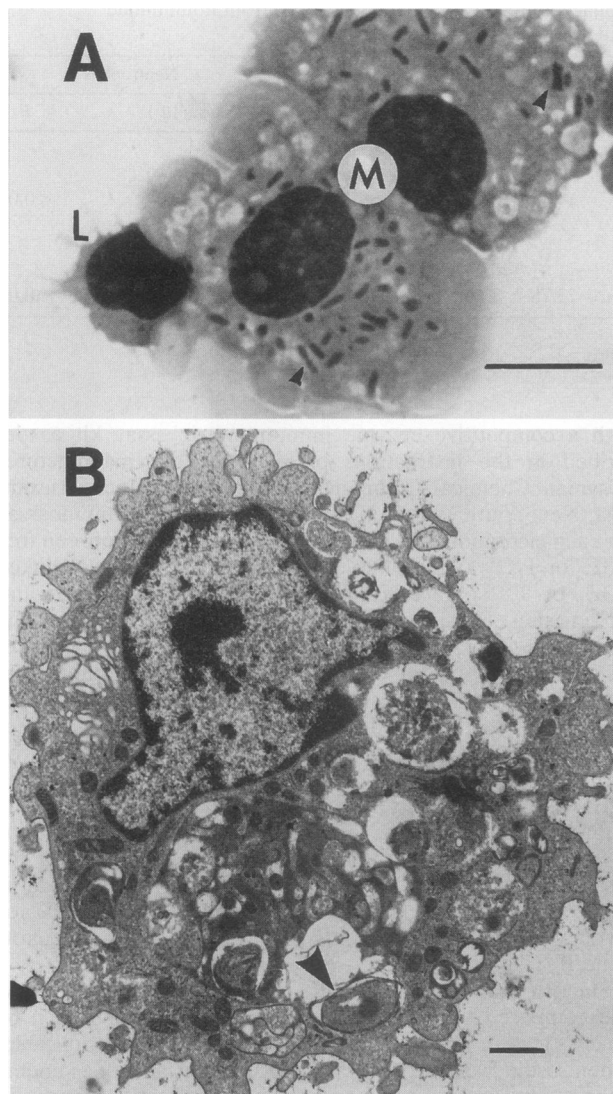
FIG. 1. (A) Light micrograph of bronchoalveolar cells from an immune animal infected for 30 min with live *P. aeruginosa*. Two alveolar macrophages (M) that have phagocytosed more than 10 bacteria (arrowheads) are easily identifiable. A lymphocyte (L) is seen in close physical interaction with one of the phagocytes. Bar, 5 µm. (B) Transmission electron micrograph of an alveolar macrophage purified from the bronchial washing of an immune rat challenged for 30 min with *P. aeruginosa*. Numerous phagocytic vacuoles, some of which contain bacteria in the process of lysis (arrowhead), are seen within the cytoplasm of the phagocyte. Bar, 1 µm.
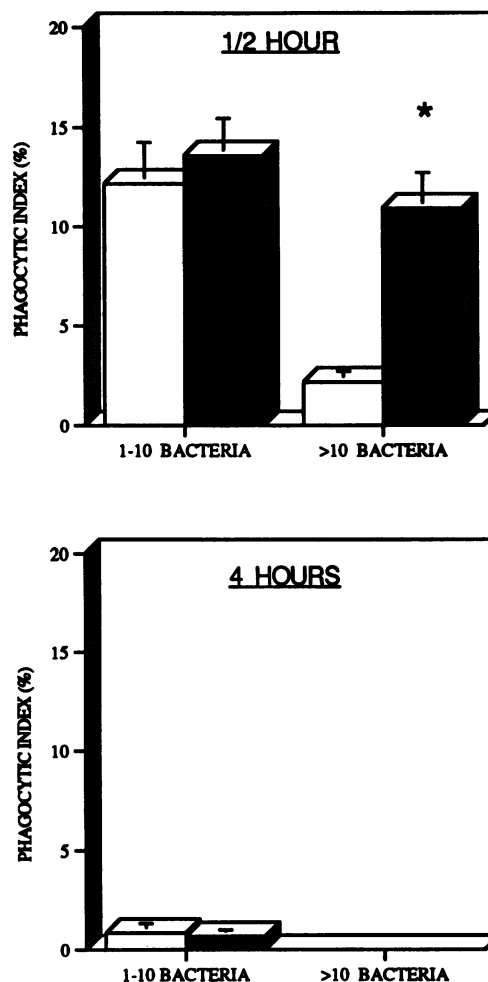

FIG. 2. Phagocytic indices in alveolar macrophages from immune (solid bars) and nonimmune (empty bars) rats challenged for 0.5 and 4 h with live *P. aeruginosa*. The figure illustrates the percentages of macrophages that have phagocytosed 1 to 10 bacteria or >10 bacteria. Values are $\bar{x}$s ± SEMs of results with six animals in each experimental group. *, $P < 0.01$ compared with values from nonimmune rats.

bacterial challenge, alveolar macrophages that had phagocytosed more than 10 bacteria were found in significantly ($P < 0.01$) greater concentrations in the BAL from immune animals than in those of nonimmune animals (Fig. 2). However, at both 30 min and 4 h, there was no difference between the percentages of macrophages that had ingested 1 to 10 microorganisms. The overall phagocytic activity of alveolar macrophages decreased significantly over time, and after 4 h of challenge, macrophages that had phagocytosed more than 10 bacteria were no longer detected in either experimental group (Fig. 2).

TNF-α and IL-1α measurements. Levels of soluble TNF-α and IL-1 measured in the BAL are illustrated in Fig. 3 and 4,

respectively. TNF-α was found in BAL after challenge with live *P. aeruginosa* but not in control animals given PBS. Concentrations of pulmonary TNF in nonimmune and immune animals were not different 30 min after intratracheal challenge, but levels rose more rapidly in the lungs of immune animals thereafter (at 4 h, nonimmune group concentration = 1.63 ± 0.21 ng per lung [$n = 8$]; immune group concentration = 4.35 ± 0.54 ng per lung [$n = 11$], $P < 0.001$ compared with nonimmune group). While TNF continued to increase in the nonimmune rat lungs after 4 h, a sharp decrease of TNF levels was measured in the immune samples ($P < 0.01$ compared with nonimmune samples at 12 h) (Fig. 3). At day 7, TNF was undetectable in BAL from immune animals. TNF-α was not detected in serum samples from control, nonimmune, and immune animals 30 min and 4 h after intratracheal inoculation (data not shown).

In BAL of immune animals, levels of soluble IL-1α remained low throughout the time of study (Fig. 4). In contrast, in BAL of nonimmune rats, concentrations of the free cytokine rose rapidly in response to the bacterial infection, until the
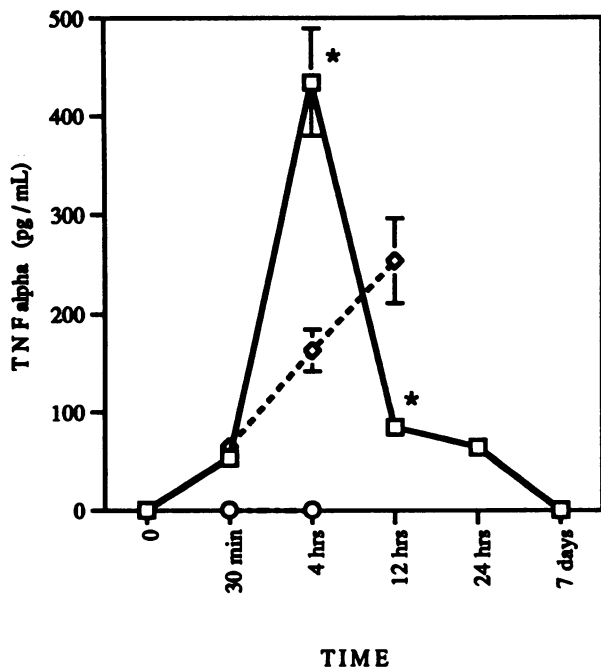
FIG. 3. Levels of soluble TNF-α calculated from the BAL of immune (□) and nonimmune (◇) rats at intervals following intratracheal inoculation with live *P. aeruginosa* and of controls (○) which received sterile PBS only. Values are x̄s ± SEMs of results with 4 to 10 animals in each experimental group. *, $P < 0.05$ compared with values from nonimmune rats.
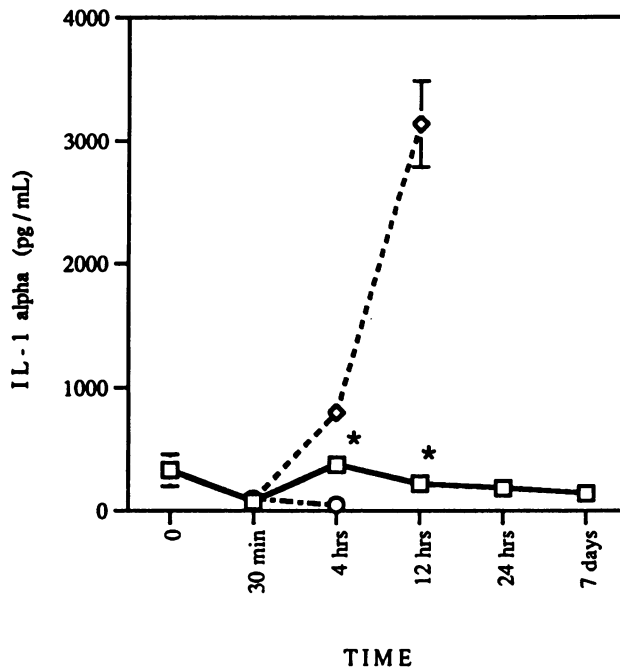
FIG. 4. Levels of soluble IL-1α calculated from the BAL of immune (□) and nonimmune (◇) animals at intervals after intratracheal inoculation with live *P. aeruginosa* and of control rats (○) which received sterile PBS only. Values are x̄s ± SEMs of results with 4 to 10 animals in each experimental group. *, $P < 0.05$ compared with values from nonimmune rats.

time of death of the animals. At 30 min, the levels of IL-1 did not differ between any groups, but at 4 h, there was more ($P < 0.05$) of the soluble cytokine in nonimmune than in immune rats and more in immune than in control samples. IL-1α was not detected in serum samples from either experimental group at 30 min or 4 h after intratracheal inoculation (data not shown).

Effect of TNF-α on bacterial proliferation in vitro. Because the results above showed that increased bacterial clearance was associated with accelerated production of soluble TNF-α in BAL of immune animals, the direct effect of this cytokine on *P. aeruginosa* was assessed. As illustrated in Fig. 5, physiological concentrations of TNF-α alone did not significantly affect the proliferation of *P. aeruginosa* in vitro.

In vivo effects of TNF-α. The effects of rTNF-α in vivo are illustrated in Table 2 and Fig. 6. Intratracheal administration of rTNF-α in physiological concentrations at the time of infection enhanced bacterial clearance (Table 2). This enhancement reached statistical significance ($P < 0.01$) with a dose of 4,000 pg of rTNF-α and was not associated with any change in total leukocyte, macrophage, or neutrophil counts in BAL 3 h postchallenge (Table 2). At this time, however, phagocytic indices of neutrophils were significantly increased in a dose-dependent fashion (Fig. 6). Only very low percentages (<5%) of alveolar macrophages contained phagocytosed bacteria in either experimental group (data not shown).

PGE₂ and TXB₂ synthesis. PGE₂ syntheses were not significantly different between immune and nonimmune animals 0.5 h after challenge (serum concentrations in nonimmune and immune animals were 2.4 ± 0.5 and 3.5 ± 1.0 ng/ml, respectively; BAL concentrations in nonimmune and immune rats were 1.7 ± 0.2 and 1.5 ± 0.1 ng/ml, respectively) or 4 h after

challenge (serum concentrations in nonimmune and immune rats were 10.0 ± 3.9 and 12.0 ± 3.3 ng/ml, respectively; BAL concentrations in nonimmune and immune rats were 1.4 ± 0.2 and 1.5 ± 0.1 ng/ml, respectively [values are x̄s ± SEMs; $n = $ five to six animals]). Similarly, syntheses of TXB₂ were not different between the two groups (data not shown).

DISCUSSION

Findings from this study demonstrate that alveolar macrophages contribute to pulmonary protection against *P. aeruginosa* in a host that had been previously immunized via the gut-associated lymphoid tissue. Enhanced bacterial clearance from the immune lung was associated with increased recruitment and phagocytic activity of alveolar macrophages coupled with accelerated production of TNF-α in the bronchoalveolar space. Physiological concentrations of rTNF-α did not affect the proliferation of *P. aeruginosa* in vitro. However, when given intratracheally at the time of infection, rTNF-α improved bacterial clearance from the lungs, an effect that was associated with a dose-dependent increase of the phagocytic activity of bronchoalveolar neutrophils. Bronchoalveolar levels of soluble IL-1α in immune animals remained low throughout the entire experimental time, and concentrations of PGE₂ or TXB₂ were not different between immune and nonimmune rats. Consistent with our previous observations (5, 7), these results confirm that intra-Peyer's-patch priming can protect the lung against infection with *P. aeruginosa*, and the present study suggests that alveolar macrophages and TNF-α are implicated in the immune cascade leading to this protection.

Macrophages play a prominent role in pulmonary protection against acute bacterial infections. *P. aeruginosa* can be phagocytosed by macrophages in the presence or absence of serum
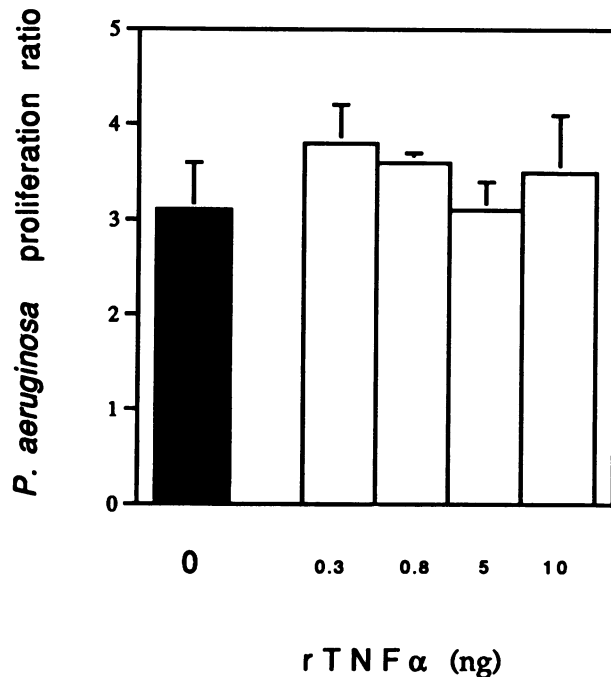
rTNFα (ng)

FIG. 5. Proliferation ratio of *P. aeruginosa* in vitro after 4 h of culture in nutrient broth to which various amounts of rTNF-α had been added. Ratios calculated from preparations containing rTNF-α (empty bars) were not significantly different from those which had none (solid bars). Values are x̄s ± SEMs of results from four experiments in each group.
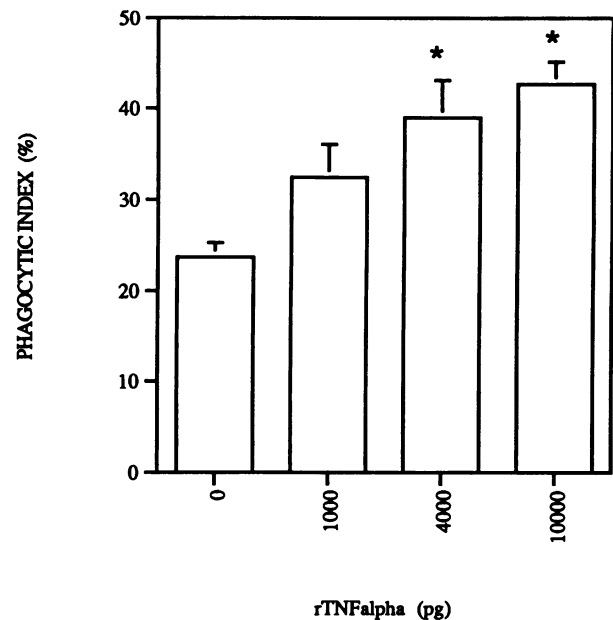

rTNFalpha (pg)

FIG. 6. Percentages of bronchoalveolar neutrophils that have phagocytosed *P. aeruginosa* 3 h postchallenge in rats that were inoculated intratracheally with 10,000, 4,000, or 1,000 pg of rTNF-α or none. Values are x̄s ± SEMs of results with eight animals in each group. *, $P < 0.01$ compared with animals that received no TNF.

opsonins (19, 27, 31). Moreover, in immunized mice, macrophages appear to be the final effector cells in protective pulmonary immunity against *Listeria monocytogenes* (36). In the present study, enhanced pulmonary clearance of *P. aeruginosa* in immune animals was associated with a significant increase in the recruitment and phagocytic activity of alveolar macrophages. In addition, the present report confirms that increased numbers and phagocytic activity of neutrophils are a critical component of pulmonary immunity against *P. aeruginosa* (5), as had been shown for *Haemophilus influenzae* (40). These observations indicate that intestinal immunization enhances the phagocytic capacity of both the macrophages and the neutrophils in the lung. The degree of specificity of this increased activity deserves to be investigated further.

The diverse functions of alveolar macrophages by far exceed the simple role of phagocytes. Among their other biological activities, they are capable of modulating the activity of other immune cells via numerous secretory products (1, 9, 27). Upon

stimulation, two of the most prominent cytokines produced by the macrophages are TNF-α and IL-1. Recent studies have associated an increased production of IL-1 and TNF-α with pathogenesis and/or protective immunity in a number of pulmonary disorders, including allergic airway disease (41), chronic obstructive pulmonary disease (39), and infections with *Mycobacterium tuberculosis* (44), *Candida albicans* (39), or *P. carinii* (16). In addition, exposure to *P. aeruginosa* stimulates mononuclear leukocytes to release IL-1 and TNF-α in vitro (32). These observations unequivocally demonstrate that production of IL-1 and TNF-α in the lung constitutes a major component of the host response to acute pulmonary disease. Accordingly, in the present study, in acutely infected nonimmune rats, pulmonary levels of soluble TNF-α and IL-1α increased consistently until the time of death. Moreover, following immunization, production of TNF-α was significantly accelerated in the lung in response to a challenge with live *P. aeruginosa*, indicating that this cytokine may mediate the increased pulmonary protection observed in a vaccinated host. In contrast, bronchoalveolar levels of soluble IL-1α remained low for reasons which require further investigation. Recent studies

TABLE 2. Bacterial recoveries (CFU) and cell counts in BAL from rats that received various doses of intratracheal rTNF-α or the vehicle alone at the time of infection[a]

rTNF-α doses (pg)	Log$_{10}$ CFU of bacteria (% clearance)[b,c]	Cell count (10^5)[b]		
		Total leukocytes	Alveolar macrophages	Neutrophils
0	8.36 ± 0.09	18.0 ± 3.3	1.31 ± 0.11	13.1 ± 3.0
1,000	8.24 ± 0.07 (25.0)	19.3 ± 9.7	1.74 ± 0.65	13.6 ± 1.3
4,000	7.96 ± 0.07* (60.3)	18.8 ± 1.5	1.82 ± 0.61	14.2 ± 1.2
10,000	8.29 ± 0.07 (15.9)	17.5 ± 1.4	1.99 ± 0.23	12.7 ± 1.2

[a] All animals were challenged for 3 h with live *P. aeruginosa*.
[b] Values are x̄s ± SEMs of results with eight animals in each experimental group.
[c] $P < 0.01$ compared with animals that received no rTNF-α.

have demonstrated that IL-4 and IL-10 increase the synthesis of IL-1 receptor antagonist and concurrently decrease that of IL-1 (8, 13, 38). Both IL-4 and IL-10 are produced by T cells, which are found in a higher state of activation in animals immunized against *P. aeruginosa* (10). Alternatively, as it has been established that LTB_4 is a potent enhancer of IL-1 production by lipopolysaccharide-stimulated monocytes (28), the significantly lower levels of LTB_4 measured in the bronchoalveolar space of immune animals from the present study (5) may also contribute to the lower concentration of IL-1α found in the same samples. Further investigations using this model will determine whether the controlled levels of IL-1α measured in the immune lung may in fact result from a higher T-cell-dependent IL-4 and/or IL-10 production and whether these changes correlate with inhibition of LTB_4 synthesis. Because IL-1 and TNF-α are potent inducers of inflammation, various methods to control their production and/or activity in situ are currently being developed in an attempt to prevent their deleterious side effects (8, 9). The study reported here shows for the first time that intestinal immunization may significantly reduce the long-term production of these cytokines at an inflammatory site. Following a live bacterial challenge, although the production of TNF-α was significantly enhanced in immune animals at 4 h, this peak was followed by a rapid drop by 12 h postchallenge. In contrast, in the lungs of nonimmune animals, levels of TNF-α consistently increased until the time of death. Moreover, levels of soluble IL-1α remained low in immune rat lungs throughout the time of study, while rising concentrations of the soluble cytokine were measured in the dying nonimmune animals. Interestingly, it has been reported that in cystic fibrosis patients colonized with *P. aeruginosa*, levels of endobronchial IL-1 and TNF-α are dramatically increased compared with control values from noninfected patients and that pulmonary inflammation correlates with cytokine concentration (34, 42). Taken together, these observations implicate both cytokines in the pathogenesis of acute *P. aeruginosa* pneumonia. The pattern of the cytokine response in immune animals strongly contrasted with that seen in nonimmune animals and correlated with improved pulmonary protection, indicating that accelerated TNF-α production and controlled levels of soluble IL-1α in the lung may constitute a rational therapeutic target for the development of an effective vaccine against *P. aeruginosa* pneumonia and possibly other pulmonary pathogens.

The role played by TNF-α in pulmonary immunity against *P. aeruginosa* was studied further. TNF-α alone had no significant effect on the proliferation of *P. aeruginosa* in vitro, which implies that this cytokine improves bacterial clearance from the lung indirectly via its immunomodulatory pathway rather than because of a direct bactericidal effect. The contrasting findings reported on the microbicidal effect of TNF-α on *P. carinii* in vitro (24) may represent a dose-dependent phenomenon since killing in the latter study could be observed only with TNF-α concentrations significantly higher than those used in the present experiments. In vitro, TNF-α has been shown to augment the phagocytic and cytotoxic activities of neutrophils (30). Gamma interferon and TNF-α also prime peritoneal macrophages for enhanced antibody-dependent cellular cytotoxicity (12), and these two cytokines potentiate the uptake and intracellular killing of opsonized *P. aeruginosa* by peritoneal macrophages (26). The latter observations are in keeping with the enhanced phagocytic indices of alveolar macrophages in vivo reported in the present investigation. That TNF-α can in part mediate the immune cascade discussed here was confirmed when the recombinant cytokine was administered intratracheally at the time of infection. Indeed, animals that re-

ceived 4,000 pg of rTNF-α saw their bacterial clearance improve, an effect associated with a dose-dependent activation of the phagocytic activity of bronchoalveolar neutrophils. However, inoculation of rTNF-α did not affect the recruitment of neutrophils into the bronchoalveolar space in vivo. Because enhanced endobronchial neutrophilic chemotaxis and chemokinesis in immunized animals have been reported previously (5), other mediators, perhaps such as granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, and/or IL-8, must be involved in the immune events seen in the present system. Granulocyte colony-stimulating factor, for example, has been shown to suppress the growth of *P. aeruginosa* in the thigh muscles of experimentally infected mice (43). Implication of other factors is further suggested by the observation that bacterial clearance was not significantly increased by 10,000 pg of endobronchial rTNF-α, despite the concurrent enhancement of phagocytic activity in bronchoalveolar neutrophils. Further investigation using this animal model will likely lead to the identification of other mediators of pulmonary immunity in intestinally vaccinated animals. In addition, such studies will help determine whether TNF-α is strictly necessary for this cascade of events to be initiated, or, alternatively, whether these other immune mediators may trigger successful protective immunity in the absence of TNF-α.

P. aeruginosa exhibits numerous immunoevasive activities (4), and one study in vitro demonstrated that elastase and alkaline protease released by the bacteria could inactivate human gamma interferon and, to a lesser extent, TNF-α (23), effects which have the potential of impairing cellular immunity. However, the present report and previous findings (5) show that pulmonary protection in immune animals correlates with enhanced recruitment and activity of alveolar macrophages and neutrophils and that these cellular events are associated with an accelerated production of TNF-α in the lung. The results imply that at least part of the immunoevasive activities displayed by *P. aeruginosa* in vitro can be overcome in vivo and that cell-mediated protection remains effective. Preliminary findings from studies using the same animal model have implicated T cells in this mechanism of protection (10), and other investigators have reported an interaction of T cells and peritoneal macrophages in vitro during cell-mediated immunity against *P. aeruginosa* (18). Similarly, a recent report showed that the host defense against intracellular infections with *M. bovis* implicates a CD4$^+$ T-cell-mediated macrophage activation which involves gamma interferon and TNF-α and requires direct physical contact between effector lymphocytes and macrophages (35). Since physical interactions between endobronchial lymphocytes and macrophages were observed in the present system (Fig. 1A), the role played by direct cell contact of CD4$^+$ T cells with phagocytes in anti-*P. aeruginosa* pulmonary immunity deserves to be investigated.

In previous studies using the same experimental system as the one described here, findings had indicated that upon bacterial challenge, levels of LTB_4, a 5'-lipoxygenase metabolite, were significantly lower in the lungs of immune animals than in those of nonimmune animals (5). As both IL-1 and TNF-α can induce PGE_2 synthesis in a variety of tissues (9) and because of the well-known immunosuppressive activities of PGE_2, it became particularly relevant to evaluate the involvement of cycloxygenase metabolites in the present experimental model. PGE_2 and TXB_2 levels did not differ between any groups, either 30 min or 4 h after infection, thus implying that these compounds are not implicated in the early postchallenge phase of pulmonary immunomodulation in immune animals challenged with *P. aeruginosa*.

In summary, adding to the well-described role of alveolar

macrophages as protective scavengers and antigen-presenting cells during an acute infection with *P. aeruginosa*, findings from the present study unequivocally demonstrate that when a host has been intestinally vaccinated, these phagocytes play a pivotal role in mediating protective pulmonary immunity via their enhanced recruitment and phagocytic activity early in the challenge infection. In addition, intestinal immunization primes the lung for accelerated production of TNF-α, which seemingly enhances phagocytosis of bacteria by neutrophils, thus further improving bacterial clearance from the lung. We postulate that following intestinal immunization with *P. aeruginosa*, pulmonary protection against a subsequent infection is achieved regardless of the immunoevasive activities of the bacteria and that this protective immunity is mediated by activated T cells and their cytokines, which stimulate alveolar macrophages and neutrophils, hence initiating a self-amplifying immunological reaction. Current studies are in the process of evaluating the implication of colony-stimulating factors in this system. Such investigations will help establish a rational basis for the development of novel immunotherapies in the treatment of pulmonary infections with *P. aeruginosa*, a research area of foremost priority in the clinical management of patients with cystic fibrosis (3).

ACKNOWLEDGMENTS

REFERENCES

1. **Adams, D. O., and T. A. Hamilton.** 1992. Macrophages as destructive cells in host defense, p. 637–653. *In* J. I. Gallin, I. M. Goldstein, and R. Snyderman (ed.), Inflammation: basic principles and clinical correlates, 2nd ed. Raven Press, Ltd., New York.
2. **Baker, P. J.** 1990. Regulation of magnitude of antibody response to bacterial polysaccharide antigens by thymus-derived lymphocytes. Infect. Immun. **58:**3465–3468.
3. **Buret, A.** 1994. *Pseudomonas aeruginosa* infections in patients with cystic fibrosis: new immunomodulatory strategies. Clin. Immunother. **2(4):**261–277.
4. **Buret, A., and A. W. Cripps.** 1993. The immunoevasive activities of *Pseudomonas aeruginosa*. Relevance for cystic fibrosis. Am. Rev. Respir. Dis. **148:**793–805.
5. **Buret, A., M. Dunkley, R. L. Clancy, and A. W. Cripps.** 1993. Effector mechanisms of intestinally induced immunity to *Pseudomonas aeruginosa* in the rat lung: role of neutrophils and leukotriene B$_4$. Infect. Immun. **61:**671–679.
6. **Chin, Y.-H., J.-P. Cai, and K. Johnson.** 1990. Lymphocyte adhesion to cultured Peyer's patch high endothelial venule cells is mediated by organ-specific homing receptors and can be regulated by cytokines. J. Immunol. **145:**3669–3677.
7. **Cripps, A. W., M. L. Dunkley, and R. L. Clancy.** 1994. Mucosal and systemic immunizations with killed *Pseudomonas aeruginosa* protect against acute respiratory infections in rats. Infect. Immun. **62:** 1427–1436.
8. **Dinarello, C. A.** 1993. Modalities for reducing interleukin 1 activity in disease. Immunol. Today **14:**260–264.
9. **Dinarello, C. A.** 1992. Role of interleukin-1 and tumor necrosis factor in systemic responses to infection and inflammation, p. 211–232. *In* J. I. Gallin, I. M. Goldstein, and R. Snyderman (ed.), Inflammation: basic principles and clinical correlates, 2nd ed. Raven Press, Ltd., New York.
10. **Dunkley, M. L., A. Buret, R. L. Clancy, and A. W. Cripps.** 1994. T-cells and IFNγ in pulmonary immunity to *Pseudomonas aeruginosa* after intestinal immunization. Am. J. Respir. Crit. Care Med. **149:**A872.
11. **Dunkley, M. L., and A. J. Husband.** 1990. Routes of priming and challenge for IgA antibody-containing cell responses in the intestine. Immunol. Lett. **26:**165–170.
12. **Fan, S., H.-G. Fehr, and D. Adams.** 1991. Activation of macrophages for ADCC *in vitro*: effects of IL-4, TNF, interferons-α/β, interferon-γ, and GM-CSF. Cell. Immunol. **135:**78–87.
13. **Galve-de Rochemonteix, B., L. P. Nicod, R. Chicheportiche, S. Lacraz, C. Baumberger, and J.-M. Dayer.** 1993. Regulation of interleukin-1ra, interleukin-1α, and interleukin-1β production by human alveolar macrophages with phorbol myristate acetate, lipopolysaccharide, and interleukin-4. Am. J. Respir. Cell Mol. Biol. **8:**160–168.
14. **Gilligan, P. H.** 1991. Microbiology of airway disease in patients with cystic fibrosis. Clin. Microbiol. Rev. **4:**35–51.
15. **Hahn, H., and S. H. E. Kaufmann.** 1981. The role of cell-mediated immunity in bacterial infections. Rev. Infect. Dis. **3:**1221–1250.
16. **Kolls, J. K., J. M. Beck, S. Nelson, W. R. Summer, and J. Shellito.** 1993. Alveolar macrophage release of tumor necrosis factor during murine *Pneumocystis carinii* pneumonia. Am. J. Respir. Cell Mol. Biol. **8:**370–376.
17. **Luo, G., D. W. Niesel, R. A. Shaban, E. A. Grimm, and G. R. Klimpel.** 1993. Tumor necrosis factor alpha binding to bacteria: evidence for a high-affinity receptor and alteration of bacterial virulence properties. Infect. Immun. **61:**830–835.
18. **Markham, R. B., G. B. Pier, J. J. Goellner, and S. B. Mizel.** 1985. In vitro T cell-mediated killing of *Pseudomonas aeruginosa*. 2. The role of macrophages and T cell subsets in T cell killing. J. Immunol. **134:**4112–4117.
19. **Mork, T., and R. E. W. Hancock.** 1993. Mechanisms of nonopsonic phagocytosis of *Pseudomonas aeruginosa*. Infect. Immun. **61:**3287–3293.
20. **Munster, A. M., and A. G. Leary.** 1977. Cell-mediated immune responses to Pseudomonas aeruginosa. Am. J. Surg. **133:**710–712.
21. **Ogle, J. D., J. G. Noel, R. M. Sramkoski, C. K. Ogle, and J. W. Alexander.** 1992. Effects of combination of tumor necrosis factor alpha and chemotactic peptide, F-MET-LEU-PHE, on phagocytosis of opsonized microspheres by human neutrophils. Inflammation **16:**57–68.
22. **Ozaki, Y., T. Ohashi, A. Minami, and S.-I. Nakamura.** 1987. Enhanced resistance of mice to bacterial infection induced by recombinant interleukin-1a. Infect. Immun. **55:**1436–1440.
23. **Parmely, M., A. Gale, M. Clabaugh, R. Horvat, and W.-W. Zhou.** 1990. Proteolytic inactivation of cytokines by *Pseudomonas aeruginosa*. Infect. Immun. **58:**3009–3014.
24. **Pesanti, E. L.** 1991. Interaction of cytokines and alveolar cells with *Pneumocystis carinii* in vitro. J. Infect. Dis. **163:**611–616.
25. **Pier, G. B., and R. B. Markham.** 1982. Induction in mice of cell-mediated immunity to *Pseudomonas aeruginosa* by high molecular weight polysaccharide and vinblastine. J. Immunol. **128:** 2121–2125.
26. **Pierangeli, S. S., and G. Sonnenfeld.** 1993. Treatment of murine macrophages with murine interferon-gamma and tumor necrosis factor-alpha enhances uptake and intracellular killing of *Pseudomonas aeruginosa*. Clin. Exp. Immunol. **93:**165–171.
27. **Reynolds, H. Y.** 1985. Phagocytic defense in the lung. Antibiot. Chemother. **36:**74–87.
28. **Rola-Pleszczynski, M., and I. Lemaire.** 1985. Leukotrienes augment interleukin 1 production by human monocytes. J. Immunol. **135:**3958–3961.
29. **Sauder, D. N., N. L. Mounessa, S. I. Katz, C. A. Dinarello, and J. I. Gallin.** 1984. Chemotactic cytokines: the role of leukocytic pyrogen and epidermal cell thymocyte-activating factor in neutrophil chemotaxis. J. Immunol. **132:**828–832.
30. **Shalaby, M. R., B. B. Aggarwal, E. Rinderknecht, L. P. Svedersky, B. S. Finkle, and M. A. J. Palladino.** 1985. Activation of human polymorphonuclear neutrophil functions by interferon-γ and tumor necrosis factors. J. Immunol. **135:**2069–2073.
31. **Speert, D. P., B. A. Loh, D. A. Cabral, and I. E. Salit.** 1986. Nonopsonic phagocytosis of nonmucoid *Pseudomonas aeruginosa* by human neutrophils and monocyte-derived macrophages is correlated with bacterial piliation and hydrophobicity. Infect. Immun. **53:**207–212.
32. **Staugas, R. E., D. P. Harvey, A. Ferrante, M. Nandoskar, and A. C. Allison.** 1992. Induction of tumor necrosis factor (TNF) and interleukin-1 (IL-1) by *Pseudomonas aeruginosa* and exotoxin A-induced suppression of lymphoproliferation and TNF, lympho-

toxin, gamma interferon, and IL-1 production in human leuko-cytes. Infect. Immun. **60:**3162–3168.

33. **Steinbeck, M. J., and J. A. Roth.** 1989. Neutrophil activation by recombinant cytokines. Rev. Infect. Dis. **11:**549–568.

34. **Suter, S., U. B. Schaad, P. Roux-Lombard, E. Girardin, G. Grau, and J.-M. Dayer.** 1989. Relation between tumor necrosis factor-alpha and granulocyte elastase-α1-proteinase inhibitor complexes in the plasma of patients with cystic fibrosis. Am. Rev. Respir. Dis. **140:**1640–1644.

35. **Sypek, J., S. Jacobson, A. Vorys, and D. J. Wyler.** 1993. Comparison of gamma interferon, tumor necrosis factor, and direct cell contact in activation of antimycobacterial defense in murine macrophages. Infect. Immun. **61:**3901–3906.

36. **Truitt, G. L., and G. B. Mackaness.** 1971. Cell-mediated resistance to aerogenic infection of the lung. Am. Rev. Respir. Dis. **104:**829–843.

37. **van der Meer, J. W. M., M. Barza, S. M. Wolff, and C. A. Dinarello.** 1988. A low dose of recombinant interleukin 1 protects granulo-cytopenic mice from lethal Gram-negative infection. Proc. Natl. Acad. Sci. USA **85:**1620–1623.

38. **Vannier, E., L. C. Miller, and C. A. Dinarello.** 1992. Coordinated antiinflammatory effects of interleukin 4: interleukin 4 suppresses interleukin 1 production but up-regulates gene expression and synthesis of interleukin 1 receptor antagonist. Proc. Natl. Acad. Sci. USA **89:**4076–4080.

39. **Vecchiarelli, A., M. Dottorini, M. Puliti, T. Todisco, E. Cenci, and F. Bistoni.** 1991. Defective candidacidal activity of alveolar mac-

rophages and peripheral blood monocytes from patients with chronic obstructive pulmonary disease. Am. Rev. Respir. Dis. **143:**1049–1054.

40. **Wallace, F. J., R. L. Clancy, and A. W. Cripps.** 1989. An animal model demonstration of enhanced clearance of nontypable *Haemophilus influenzae* from the respiratory tract after antigen stimulation of gut-associated lymphoid tissue. Am. Rev. Respir. Dis. **140:**311–316.

41. **Watson, M. L., D. Smith, A. D. Bourne, R. C. Thompson, and J. Westwick.** 1993. Cytokines contribute to airway dysfunction in antigen-challenged guinea pigs: inhibition of airway hyperreactivity, pulmonary eosinophil accumulation, and tumor necrosis factor generation by pretreatment with an interleukin-1 receptor antagonist. Am. J. Respir. Cell Mol. Biol. **8:**365–369.

42. **Wilmott, R. W., J. T. Kassab, P. L. Kilian, W. R. Benjamin, S. D. Douglas, and R. E. Wood.** 1990. Increased levels of interleukin-1 in bronchoalveolar washings from children with bacterial pulmonary infections. Am. Rev. Respir. Dis. **142:**365–368.

43. **Yasuda, H., Y. Ajiki, T. Shimozato, M. Kasahara, H. Kawada, M. Iwata, and K. Shimizu.** 1990. Therapeutic efficacy of granulocyte colony-stimulating factor alone and in combination with antibiotics against *Pseudomonas aeruginosa* infections in mice. Infect. Immun. **58:**2502–2509.

44. **Zhang, Y., M. Doerfler, T. C. Lee, B. Guillemin, and W. N. Rom.** 1993. Mechanisms of stimulation of interleukin-1β and tumor necrosis factor-α by *Mycobacterium tuberculosis* components. J. Clin. Invest. **91:**2076–2083.