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Role of Tumor Necrosis Factor Alpha in Innate Resistance to Mouse Pulmonary Infection with *Pseudomonas aeruginosa*

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In the present study, we have investigated the mechanisms underlying mouse resistance to endobronchial infection with *Pseudomonas aeruginosa* enmeshed in agar beads. This was done by monitoring macrophage activation-associated gene expression in lung and alveolar cells harvested from resistant (BALB/c) and susceptible (DBA/2, C57BL/6, and A/J) strains of mice over the course of infection with *P. aeruginosa*. Interleukin-1α, interleukin-1β, macrophage inflammatory protein-1α, JE, and tumor necrosis factor alpha (TNF-α) mRNA expression levels were up-regulated in all strains of mice during the early phase of the infection. The level of TNF-α mRNA expression was increased to a greater extent in resistant BALB/c mice than in susceptible DBA/2, C57BL/6, and A/J strains of mice. This observation paralleled a higher secretion of TNF-α into the alveolar space of BALB/c mice at 3 and 6 h postinfection. The concentration of TNF-α released in alveoli returned to basal levels within 24 h of infection in mice of all strains, even though the TNF-α mRNA expression remained high until 3 days after infection. In vivo treatments with either anti-murine TNF-α monoclonal antibodies or with aminoguanidine significantly increased the number of *P. aeruginosa* bacteria detected in the lungs of resistant mice at 3 days postinfection. Overall, these findings indicate that both TNF-α and nitric oxide exert a protective role in response to pulmonary infection with *P. aeruginosa*.

Pulmonary infection with *Pseudomonas aeruginosa* is a major factor contributing to the morbidity and mortality of patients with cystic fibrosis (CF). Chronic infection with *P. aeruginosa* has been associated with progressive obstruction of the small airways, deteriorating lung function most likely caused by repeated cycles of pulmonary infection, and an excessive host inflammatory response. The clinical manifestations of CF arise from a defect at the CF transmembrane conductance regulator (CFTR) locus. Different mutations in the CFTR gene may account for the variable clinical expression of CF pulmonary disease (22, 37). Nevertheless, striking heterogeneity exists in the severity of *P. aeruginosa*-induced pulmonary disease in CF patients with identical CFTR defects, thereby implicating other loci in the host response to *P. aeruginosa* infection (17, 37, 38).

To study the genetic factors involved in the host resistance to *P. aeruginosa* infection, Morissette and colleagues (27) have developed a murine model for endobronchial infection with *P. aeruginosa*. When mice of different strains were infected intratracheally with a low dose (1×10^4 to 5×10^4) of *P. aeruginosa* cells enmeshed in agar beads, a strain-dependent variation in mouse resistance to infection was observed. Mice of the BALB/c strain initiated a strong clearance of the bacteria 3 days postinfection and were typed as resistant to infection. In contrast, mice of the DBA/2 strain were the most susceptible to infection and had a high bacterial load and a high mortality at 3 days postinfection. Mice of the A/J and C57BL/6 strains were equally susceptible to infection as those of the DBA/2 strain in terms of the number of bacteria detected in the lungs early after infection but not in their death rate, which was lower than that of the DBA/2 strain. With this model of infection, the present study is aimed at elucidating the mechanisms underlying the resistance and susceptibility to *P. aeruginosa* infection. Preliminary lines of evidence suggest that phagocytes recruited to the site of infection play an important role in host resistance to this infection (27, 29, 40). Consequently, several cytokines associated with macrophage activation for bactericidal function were monitored in the lungs of susceptible and resistant mice over the course of *P. aeruginosa* infection in the present study. Interleukin-1α (IL-1α), IL-1β, macrophage inflammatory protein-1α (MIP-1α), JE, and tumor necrosis factor alpha (TNF-α) mRNA expression levels in alveolar and lung cells as well as IL-1α, IL-1β, and TNF-α protein secretion in alveoli were up-regulated following *P. aeruginosa* infection. Among these cytokines, TNF-α was of particular interest since the level of TNF-α mRNA expression and the concentration of TNF-α protein secreted into bronchoalveolar lavage fluid (BAL) of infected mice was associated with resistance to infection. In fact, this proinflammatory cytokine plays a key role in resistance to several other pulmonary infections (6, 9, 18) but, when produced in excess, also leads to shock, lung damage, fibrosis, and tissue dysfunction (19, 21, 34, 44). Thus, it was of interest to clarify the exact role of TNF-α in the host response to endobronchial infection with *P. aeruginosa*. This was done by measuring the effect of TNF-α depletion on the number of *P. aeruginosa* bacteria recovered from the lungs of resistant and susceptible mice at 3 days postinfection. In this study, we established that TNF-α plays a protective role in resistance to infection with *P. aeruginosa* and we proposed a mechanism by which TNF-α may exert its action.

MATERIALS AND METHODS

Mice. Male 8- to 12-week-old BALB/cAnN, C57BL/6N, DBA/2N (Harlan Sprague Dawley, Indianapolis, Ind.), and A/J(Cr) (National Cancer Institute, Frederick, Md.) mice were maintained under specific-pathogen-free conditions until infection.

Anti-murine TNF-α MAb. Anti-TNF-α monoclonal antibody (MAb)-containing ascites fluids were collected from pristane-primed nude BALB/c mice (Harlan Sprague Dawley) inoculated intraperitoneally (i.p.) 7 days previously with 10^7 XT22 hybridoma cells (American Type Culture Collection, Rockville, Md.). The

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TNF-neutralizing activity in ascites was measured in a standard TNF bioassay. Briefly, L929 cells (American Type Culture Collection) treated with actinomycin D (2 μg/ml; Sigma Chemical Co., St. Louis, Mo.) were incubated overnight with serial dilutions of ascites and with 10 U of recombinant TNF-α (Genzyme, Boston, Mass.). The viability of the L929 cells was evaluated after incubation with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma). One neutralizing unit was defined as the reciprocal of the highest ascites dilution that neutralized 50% of the TNF-α-mediated cytotoxic activity on L929 cells.

Infection of mice with *P. aeruginosa*. Mice were infected intratracheally with a clinical mucoid isolate of *P. aeruginosa* 508 (provided by Jacqueline Lagacé, University of Montreal, Montreal, Canada) enmeshed in agar beads as described previously (27). Briefly, a log-phase bacterial suspension diluted in warm (52°C) Trypticase soy agar was added to heavy mineral oil (Fisher Scientific, Fair Lawn, N.J.; 52°C) and stirred vigorously for 6 min at 20°C, and the mixture was cooled with ice for 10 min. The bacterium-containing beads formed were smaller than 200 μm in diameter. They were isolated by centrifugation at 9,000 × *g* for 20 min (4°C), washed extensively, and suspended in phosphate-buffered saline (PBS; ICN Biomedicals Inc., Costa Mesa, Calif.). The density of viable *P. aeruginosa* bacteria enmeshed in agar beads was determined by plating serial dilutions of homogenized bead suspension onto plates containing Trypticase soy agar medium. The bacterium-containing bead suspension was diluted immediately before infection at a density of 2×10^5 to 1×10^6 CFU/ml.

Mice were anesthetized with a mixture of ketamine hydrochloride (75 mg/kg of body weight; MTC Pharmaceuticals, Cambridge, Ontario, Canada) and xylazine hydrochloride (30 mg/kg of body weight; Bayvet Division, Chemagro Limited, Etobicoke, Ontario, Canada) injected intramuscularly. The trachea was exposed by ventral midline cervical incision, and 50 μl of the bead suspension followed by 50 μl of air was inoculated into the lungs through a 22-gauge intravenous catheter (Critikon, Tampa, Fla.) inserted into the trachea. After inoculation, the incision was closed by suture. Animals did not develop wound infections, and healing occurred within 2 to 3 days.

BAL. The alveoli of infected mice or normal age-matched controls sacrificed by inhalation of CO_2 were washed seven times with 1 ml of PBS via the cannulated trachea. The volume of BAL recovered was approximately 5 ml.

CFU. The lungs excised from infected mice were homogenized for 30 s (speed 4, homogenizer PT10135; Brinkmann Instruments, Inc., Mississauga, Ontario, Canada) in 10 ml of PBS, and serial 10-fold dilutions of lung homogenates were plated onto petri dishes containing Trypticase soy agar. The number of viable *P. aeruginosa* bacteria per lung was counted after incubation overnight at 37°C.

TNF-α assay. The concentration of TNF-α in BAL samples of infected and normal mice was determined in a double sandwich enzyme-linked immunosorbent assay (ELISA) as described by Sheehan and colleagues (39). Briefly, 96-well ELISA microplates were coated overnight (4°C) with hamster anti-murine TNF MAb (Genzyme). Plates were then washed and incubated sequentially with various dilutions of BAL samples, polyclonal rabbit anti-murine TNF-α antiserum (kindly provided by Mary M. Stevenson, Montreal General Hospital, Montreal, Quebec, Canada), and peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad, Hercules, Calif.). The intensity of the colorimetric reaction was measured by spectrometry at 405 nm. The level of TNF-α in BAL samples was calculated with reference to a standard curve established with recombinant TNF-α (Genzyme).

Extraction of total cellular RNA. Cells obtained from BAL samples of infected mice were solubilized in 0.2 ml of 4 M guanidine isothiocyanate solution (Gibco BRL, Gaithersburg, Md.), and total RNA was isolated by a single-step acidic phenol extraction as described elsewhere (20). For RNA extraction from lung cells, the tissue was homogenized in 5 ml of guanidine isothiocyanate solution and centrifuged on a cesium chloride gradient.

Northern (RNA) blot analysis. RNA (15 to 20 μg) extracted from total lung cells was separated on a 1.2% agarose gel containing 2.2 M formaldehyde and transferred to a Nytran nylon membrane (Schleicher & Schuell, Inc., Keene, N.H.) by capillary action followed by UV cross-linking. RNA was then hybridized overnight at 42°C with probes labelled with [α-^{32}P]dCTP (Amersham Canada, Oakville, Ontario, Canada) by use of a nick translation system (Gibco-BRL). TNF-α cDNA probe (1.3 kb) was kindly provided by A. Cerami (Rockefeller University, New York, N.Y.). The murine glyceraldehyde-3-phosphate dehydrogenase (GADPH) probe was generated by PCR amplification of murine cDNA with sense (5′ CCC TTC ATT GAC CTC AAC TAC ATGG 3′) and antisense (5′ AGT CTT CTG GGT GGC ATG GAT GG 3′) primers. The resulting DNA fragment (456 bp) was blunt ended and ligated into pGEM3Z vector (Promega Corporation, Madison, Wis.). The identity of the insert was verified by restriction analysis and sequencing. Hybridized filters were washed three times in 2× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at 42°C and in 0.1× SSC buffer–0.1% SDS at 55°C. The filters were then exposed to X-Omat AR films (Kodak, Rochester, N.Y.), and the relative optical density of TNF-α mRNA was analyzed with a SciScan 5000 scanner (U.S. Biochemicals, Cleveland, Ohio) in reference to GADPH mRNA expression.

Reverse transcription-PCR. Total RNA extracted from alveolar cells was transcribed into cDNA with Moloney murine leukemia virus reverse transcriptase (U.S. Biochemicals; 1 h at 37°C). The enzyme was then heat inactivated (95°C) and diluted with water. The amount of cDNA in each sample was normalized on the basis of the level of GADPH mRNA expression by quantitation with high-resolution optical SciScan 5000 densitometry. Gene-specific cDNA was then amplified by PCR with *Taq* polymerase (Promega, Southampton, United Kingdom), [α-^{32}P]dCTP (Amersham), and primers specific for TNF-α or GADPH (Table 1). Primers were designed in our laboratory, synthesized at Sheldon Biotechnology Centre (Montreal, Quebec, Canada), and purified by high-performance liquid chromatography. The PCR products obtained after 25 cycles of amplification were run on 4.5% polyacrylamide gels. Fixed and dried gels were then exposed to X-Omat AR film. The level of TNF-α expression was normalized to GADPH gene expression by densitometric analysis.

Statistical analyses. The strain-dependent variations in TNF-α production (see Fig. 3a) and in the number of *P. aeruginosa* bacteria (see Fig. 3b) recovered from the lungs following infection were analyzed by analysis of variance and Student-Newman-Keuls tests for independent samples. The numbers of *P. aeruginosa* bacteria detected in the lungs of infected mice treated with either anti-TNF-α MAb (see Fig. 4a) or aminoguanidine (see Fig. 4b) were compared with those of untreated infected controls by a nonparametric Mann-Whitney U test.

RESULTS

TNF-α mRNA expression in lung and alveolar cells collected from resistant and susceptible mice over the course of infection with *P. aeruginosa*. In an attempt to explore the mechanisms underlying host resistance to pulmonary infection with *P. aeruginosa*, several genes encoding cytokines associated with macrophage activation by bacteria were studied. The levels of expression of IL-1α, IL-1β, MIP-1α, JE, and TNF-α were measured in the lung and alveolar cells harvested from mice infected intratracheally with *P. aeruginosa* enmeshed in agar beads. The mRNA expression of these genes was monitored in *P. aeruginosa*-resistant (BALB/c) and *P. aeruginosa*-susceptible (DBA/2, A/J, and C57BL/6) inbred strains of mice over a period ranging from 3 h to 14 days postinfection. IL-1α, IL-1β, JE, and TNF-α mRNA levels were up-regulated early after endobronchial infection with *P. aeruginosa* in all strains of mice tested (data not shown). Since the level of mRNA expression of TNF-α but not those of IL-1α, IL-1β, MIP-1α, or JE were increased to a greater extent in resistant mice, the involvement of TNF-α during the course of infection was investigated further. The increased TNF-α mRNA level found in the lung cells appeared to be associated with the early host response to infection, since it peaked 3 to 24 h postinfection, after which it declined in all strains of mice tested (Fig. 1). The level of TNF-α mRNA in the lung cells (Fig. 1) as well as in the alveolar cells (Fig. 2) harvested after 3, 6, and 24 h following infection with *P. aeruginosa* was higher in BALB/c mice than in DBA/2, A/J, or C57BL/6 mice. Overall, these results suggest that resistance to endobronchial infection with *P. aeruginosa* may be associated with higher levels of TNF-α mRNA expression.

Levels of TNF-α released in the alveolar space of susceptible and resistant strains of mice following infection with *P. aeruginosa*. To establish whether the up-regulated TNF-α mRNA expression found in the lung and alveolar cells from *P. aeruginosa*-infected mice was accompanied by enhanced TNF-α protein secretion, the concentration of TNF-α was measured in BAL samples harvested from the susceptible and resistant mice 3, 6, 24, and 72 h postinfection. As shown in Fig. 3a, intratracheal infection with a low dose of *P. aeruginosa* enmeshed in agar beads induced the release of TNF-α in alveoli in the four strains of mice tested. TNF-α production peaked between 3 and 6 h postinfection, after which it returned progressively to the basal level. The amount of TNF-α detected at 3 and 6 h postinfection was significantly higher ($P < 0.01$) in the resistant BALB/c mice than in the susceptible DBA/2, C57BL/6, and A/J strains of mice (Fig. 3a). Low levels of TNF-α were found in BAL samples harvested from control mice 3 h after inoculation of agar beads alone (data not shown). Nevertheless, the amount of TNF-α detected in BAL

TABLE 1. Primer sequences and experimental conditions used for reverse transcription-PCR

mRNA	Sense primer, 5′ to 3′	Antisense primer, 5′ to 3′	Size of PCR products (bp)	PCR conditions
TNF-α	CAC ACT CAG ATC ATC TTC TCA AAA	GCA ATG ACT CTA AAG TAG ACC TGC	468	94°C for 40 s, 60°C for 40 s, 72°C for 90 s
GADPH	CCC TTC ATT GAC CTC AAC TAC ATG G	AGT CCT CTG GGT GGC ATG GAT GG	456	94°C for 40 s, 65°C for 60 s, 72°C for 60 s

samples of these mice remained significantly lower than that found in mice inoculated with bacterium-containing beads. Furthermore, the TNF-α protein secretion detected in control mice was not accompanied by any increase in TNF-α mRNA expression in lung and alveolar cells. The slight TNF-α protein secretion observed following injection of agar beads alone may thus result from the release of constitutively expressed low levels of TNF-α mRNA or from the release of TNF-α protein present in the intracellular stores.

As shown in Fig. 3b, BALB/c mice were able to control the infection with *P. aeruginosa* from the third day following the infection. At this time, the level of TNF-α protein detected in the BAL samples was decreased substantially in all strains of mice tested. These data prompted us to postulate that higher TNF-α levels produced during the early phase of *P. aeruginosa* infection in the lungs of BALB/c mice might have a protective effect against the infection.

Role of TNF-α in resistance to endobronchial infection with *P. aeruginosa*. In an attempt to test this hypothesis and to elucidate the role of TNF-α in pulmonary infection with *P. aeruginosa*, we have evaluated the effect of in vivo depletion of TNF-α on the course of infection with *P. aeruginosa*. Treatment i.p. with 2×10^4 neutralizing units of rat anti-murine TNF-α MAb 2 and 24 h before as well as 24 h after infection with *P. aeruginosa* significantly ($P < 0.05$) increased the numbers of *P. aeruginosa* bacteria recovered from the lungs of BALB/c mice at 72 h postinfection (Fig. 4a). The numbers of bacteria detected in the lungs of susceptible DBA/2 mice, however, remained almost unchanged following treatment with anti-TNF-α MAb. Administration of equivalent quantities (based on protein concentration) of normal rat immunoglobulins did not significantly affect the number of CFU found in the lungs of either susceptible or resistant mice infected with *P. aeruginosa*. The treatment with anti-TNF-α MAb was shown to deplete over 85% of the TNF-α detected in the BAL samples of BALB/c mice at 3 h postinfection as measured by ELISA and TNF-α bioassay (data not shown).

The increased susceptibility to *P. aeruginosa* infection observed in BALB/c mice depleted of TNF-α could not be attributed to a reduced number of cells recruited to the site of infection. Indeed, the total number of alveolar cells detected in BAL samples from BALB/c mice 3 days after infection was not decreased following treatment with anti-TNF-α MAb (data not shown). The absolute numbers of macrophages, polymorphonuclear cells (PMN), or lymphocytes in alveoli also remained unaffected in BALB/c mice after in vivo TNF-α depletion.

Involvement of nitric oxide in host response to endobronchial infection with *P. aeruginosa*. Since NO and reactive nitrogen intermediates (RNI) are known to be highly potent antimicrobial factors that can be induced by TNF-α (10, 13, 24), the levels of RNI released in BAL were monitored in resistant BALB/c and susceptible DBA/2 mice over the course of *P. aeruginosa* infection. Nitrites and nitrates were barely detectable in BAL and serum samples harvested from BALB/c and DBA/2 mice at 3, 6, 24, and 72 h postinfection (data not shown). A slight increase in inducible NO synthase (iNOS) mRNA expression was observed in lung cells harvested from susceptible or resistant mice from 3 days postinfection (data not shown). An enhanced iNOS mRNA expression was detected in alveolar cells at 24 and 72 h postinfection, although it was not associated with resistance to *P. aeruginosa* infection (data not shown). To establish more clearly the role of NO in the host response against *P. aeruginosa* infection, iNOS was inhibited with aminoguanidine given i.p. twice daily starting 2 days before infection with *P. aeruginosa* enmeshed in agar beads. This treatment significantly impaired ($P < 0.02$) the

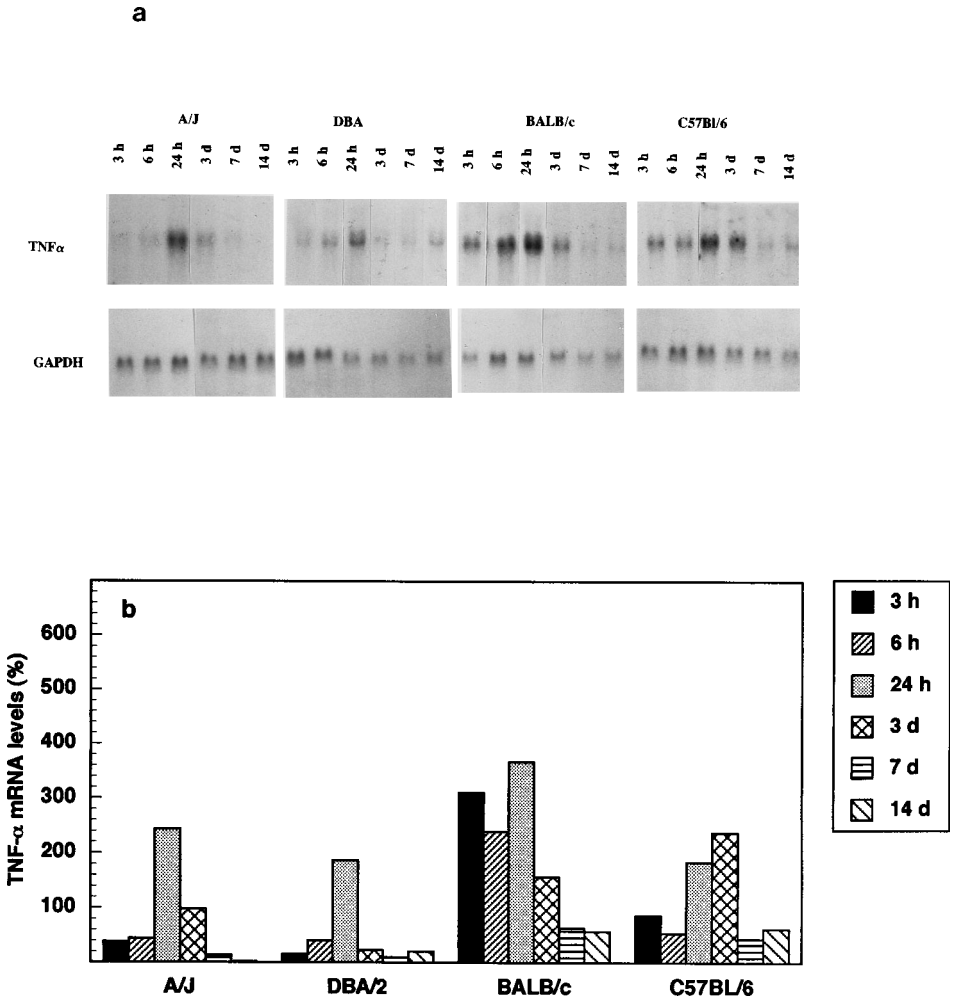

FIG. 1. TNF-α mRNA expression in lung cells of susceptible and resistant strains of mice over the course of infection with *P. aeruginosa*. Total RNA was extracted from lung cells of susceptible (A/J, DBA/2, and C57BL/6) or resistant (BALB/c) mice infected intratracheally with *P. aeruginosa* at various times postinfection. (a) The levels of TNF-α and GADPH mRNA expression were evaluated by Northern blot analysis. (b) The levels of TNF-α mRNA expression were quantified by densitometric analysis and normalized to the level of GADPH mRNA expression.

ability of BALB/c mice to control the growth of *P. aeruginosa* as detected at 3 days postinfection (Fig. 4b). The number of viable *P. aeruginosa* bacteria detected in the lungs of susceptible DBA/2 mice at 3 days postinfection remained unchanged after treatment with aminoguanidine. Impairment of NO production upon administration of aminoguanidine was controlled by measuring the level of RNI in BAL and serum samples from lipopolysaccharide (LPS)-primed BALB/c and DBA/2 mice treated with aminoguanidine. Taken together, these results suggest that both NO and TNF-α play an important role in host resistance to endobronchial infection with *P. aeruginosa* enmeshed in agar beads.

DISCUSSION

In this study, we report that intratracheal infection of mice with *P. aeruginosa* enmeshed in agar beads leads to up-regulation of TNF-α mRNA expression. This up-regulation was associated with an enhanced secretion of TNF-α protein into the alveolar space at 3 and 6 h postinfection. TNF-α protein concentration returned to basal levels within 24 h postinfection in mice of all strains, even though the level of mRNA expression was still high 3 days after infection. This suggests post-transcriptional regulation. The acute TNF-α release detected early after *P. aeruginosa* infection is consistent with earlier studies which have also demonstrated that TNF-α is produced within a few hours in the alveolar space of rodents following lung infection with *Legionella pneumophila* (5), *Klebsiella pneumoniae* (31), *Staphylococcus aureus* (31), *Histoplasma capsulatum* (42), and *Pneumocystis carinii* (18) and returns to the basal level within 24 h postinfection. Infection of mice with influenza virus was, however, shown to delay the TNF-α release (45). In our model, the observation that higher levels of both TNF-α mRNA expression and TNF-α protein secretion in mice infected with *P. aeruginosa* were associated with host resistance to infection suggested that TNF-α may represent an important genetically regulated early defense signal crucial for the outcome of acute infection with *P. aeruginosa*. This hypothesis was further supported by the finding that in vivo depletion of TNF-α markedly impaired the ability of resistant BALB/c mice to control infection with *P. aeruginosa*. The fact that treatment with anti-TNF-α MAb did not exert any detectable effect in susceptible DBA/2 mice was not surprising given the low amount of TNF-α produced in alveoli of these mice over the course of infection. To our knowledge, these experiments are the first to directly address the role of TNF-α in pulmonary infection with *P. aeruginosa* by in vivo depletion. A protective

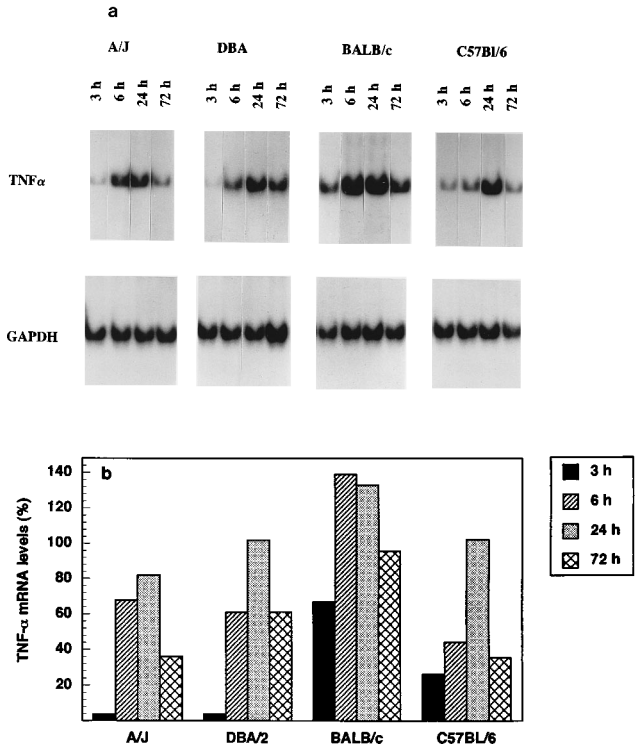

FIG. 2. Comparison of TNF-α mRNA expression levels in alveolar cells of susceptible and resistant mice infected with *P. aeruginosa*. Alveolar cells were collected from susceptible (A/J, DBA/2, and C57BL/6) or resistant (BALB/c) mice 3 to 72 h after intratracheal infection with *P. aeruginosa*. The levels of TNF-α mRNA expression were analyzed by reverse transcription-PCR (a) and normalized by densitometric analysis to the level of GADPH mRNA expression in the same experimental sample (b).

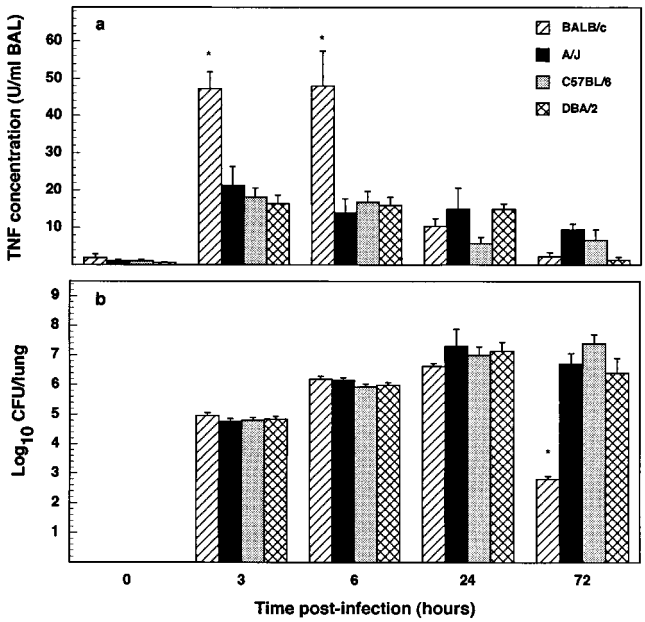

FIG. 3. Strain-dependent variations in TNF-α produced in alveoli and in the number of *P. aeruginosa* cells detected in the lungs following infection. (a) TNF-α concentration was measured in BAL samples collected from BALB/c, A/J, C57BL/6, and DBA/2 mice 3 to 72 h following intratracheal infection with 1.8×10^4 *P. aeruginosa* bacteria. (b) The $\log_{10}$ CFU detected in the lungs of infected mice at each time point is shown. Results are expressed as the means of three to six individual mice ± standard errors of the means and were repeated once. *, significant ($P < 0.01$) difference in TNF-α concentration or bacterial load detected in BALB/c mice compared with those in the A/J, C57BL/6, and DBA/2 strains of mice.

role of TNF-α in *P. aeruginosa* infection in rats was reported previously by Buret and colleagues (7), who observed that a single intratracheal inoculation of recombinant TNF-α at the time of infection decreased the number of *P. aeruginosa* bacteria found in the lungs of infected rats. The importance of TNF-α in other pulmonary infections was demonstrated by in vivo treatments with anti-TNF-α MAb or with recombinant TNF-α (6, 9, 42). The protective effect of human recombinant TNF-α was also documented in the peritoneal cavity of mice infected i.p. with *P. aeruginosa* (29). On the other hand, a number of reports have also demonstrated that high levels of TNF-α sustained over a prolonged period may cause severe lung damage (36, 44). Furthermore, the progression of experimentally induced pulmonary fibrosis in mice could be prevented by treatment with anti-TNF-α antibody and was markedly increased by continuous infusions of mouse recombinant TNF-α (34). Overall, the TNF-α could play a beneficial role in acute bacterial infections, but it must be tightly regulated to avoid tissue destruction later during the course of chronic infections. In our model, the transient release of TNF-α during the early phase of pulmonary infection with *P. aeruginosa* would thus be consistent with this cytokine being a host-mediated response exerting innate resistance to infection rather than a mediator of lung pathogenesis.

TNF-α is known to enhance host resistance to infections by regulating a cascade of specific and nonspecific immunological responses. At present, the mechanism by which TNF-α is involved in host resistance to endobronchial infection with *P. aeruginosa* remains to be elucidated. One possibility is that TNF-α could exert a lethal effect on *P. aeruginosa*, as reported previously for *Pneumocystis carinii* (33). Our results suggest that a direct toxic effect of TNF-α is unlikely to be of significant importance in host resistance to infection with *P. aeruginosa*. Indeed, TNF-α production peaked within 3 to 6 h after infection in resistant BALB/c mice, whereas the ability of these mice to control the infection was observed only from 3 days postinfection. Moreover, Buret and colleagues (7) have demonstrated previously that the in vitro proliferation of *P. aeruginosa* is not affected by the addition of various concentrations of TNF-α. This cytokine is also known to promote the recruitment of PMN and macrophages to the site of infection (19, 46). This effect was shown to be associated with the capacity of TNF-α to up-regulate the expression of adhesion molecules on endothelial and epithelial cells as well as PMN (28, 43), to stimulate the secretion of chemotactic factors by epithelial cells and phagocytes (41), or to act directly as a chemoattractant (25). Since we have established that the release of TNF-α preceded the influx of inflammatory cells into the lungs of mice infected with *P. aeruginosa* (27), it is conceivable that TNF-α plays a role in the recruitment of inflammatory cells. We have addressed this possibility and found that the numbers of alveolar PMN, macrophages, and lymphocytes in BAL samples of infected BALB/c mice were not decreased following in vivo treatment with anti-TNF-α MAb. Furthermore, it was shown previously that an intratracheal inoculation of recombinant TNF-α with *P. aeruginosa* did not alter the number of leukocytes attracted to the lungs of infected rats (7). These findings thus exclude the possibility that the protective effect of TNF-α is exerted through the recruitment of inflammatory cells to the site of infection.

Finally, TNF-α can enhance host resistance to infection by regulating several phagocyte functions involved in bactericidal activity. A number of in vitro studies have established that infected macrophages activated with gamma interferon (IFN-γ)

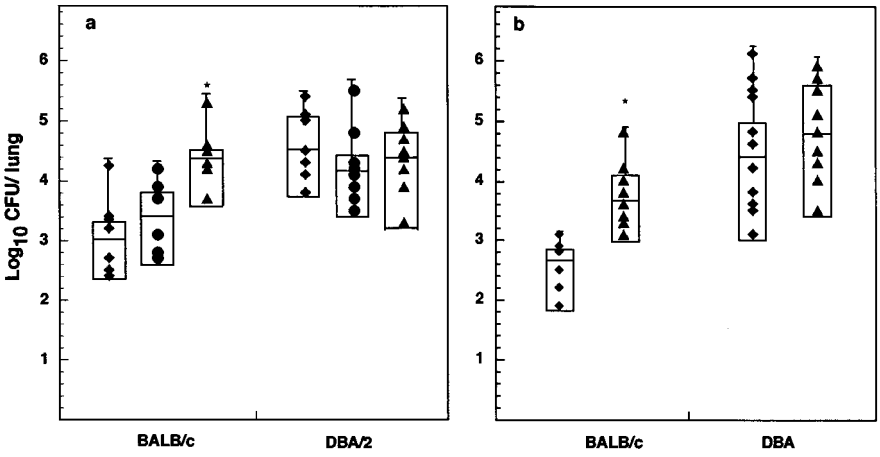

FIG. 4. Effect of TNF-α depletion or iNOS inhibition on the number of *P. aeruginosa* bacteria recovered from the lungs of infected BALB/c and DBA/2 mice. (a) Resistant (BALB/c) and susceptible (DBA/2) mice were inoculated i.p. with 2×10^4 neutralizing units of anti-TNF-α MAb (clone XT22) (▲), with an equal amount of control rat immunoglobulins (based on protein concentration) (●), or with 0.5 ml of PBS (◆). Treatments were given 24 and 2 h before as well as 24 h after intratracheal infection with 3.5×10^4 *P. aeruginosa* bacteria. (b) In another series of experiments, BALB/c and DBA/2 mice were treated with 0.5 ml of PBS (◆) or with 1.25 mg of aminoguanidine (▲) injected i.p. twice daily, starting 2 days before intratracheal infection with 1.1×10^4 *P. aeruginosa* bacteria. The number of CFU in the lungs of mice was measured 3 days after infection by plating serial 10-fold dilutions onto Trypticase soy agar. Results obtained from 6 to 11 mice are presented as dots, medians, and interquartiles. The experiment illustrated in panel a was done three times, while that in panel b was done twice. *, statistically significant ($P < 0.05$) difference compared with control PBS or control normal immunoglobulin treatments.

produce large amounts of NO and its oxidative metabolites, nitrites and nitrates. It was shown that IFN-γ and the microorganisms interact synergistically to stimulate the endogenous release of TNF-α by macrophages. TNF-α would then act in an autocrine manner, amplifying the synthesis and release of NO and RNI by IFN-γ-primed macrophages (10, 13, 24). In vitro studies have shown that RNI can be toxic for several microorganisms, including parasites (14, 24, 47), bacteria (1, 3, 4), viruses (16), and fungi (2). Evidence that NO is a key factor in host response against several pathogens was provided in animals treated with either N^G-monomethyl-L-arginine (12, 23) or aminoguanidine (4, 15a), two potent inhibitors of NO synthase. Although the role of NO in pulmonary infections remains poorly understood, it is conceivable that NO could act as a potential mediator of resistance to lung infection with *P. aeruginosa*, inasmuch as alveolar and interstitial pulmonary macrophages have the capacity to produce NO (32, 35). Furthermore, LPS, an important constituent of the outer membrane of gram-negative bacteria, has been shown to act in synergy with IFN-γ to stimulate the synthesis of NO (10, 14). We detected only low levels of RNI in BAL samples of resistant BALB/c mice and susceptible DBA/2 mice between 3 and 72 h following infection with *P. aeruginosa*. These findings do not, however, exclude the possibility that RNI are produced in the lungs of infected animals in that they may be utilized very rapidly, rendering their detection very difficult. Since iNOS mRNA is tightly regulated at the posttranscriptional level (30), the analysis of its expression during the course of infection would be difficult to interpret. The role of NO in resistance to *P. aeruginosa* infection was thus addressed directly in vivo by inhibiting the production of NO. Aminoguanidine was chosen for this purpose because it was shown to be less toxic to mice than N^G-monomethyl-L-arginine. In addition, aminoguanidine selectively inhibits the cytokine-inducible isoform of NO synthase which is known to be responsible for the production of high levels of NO involved in bactericidal activity (26). The dose of aminoguanidine (50 mg/kg of body weight of mice) administered to *P. aeruginosa*-infected mice was selected to yield an efficient inhibition of NO production without any apparent toxic effects. Aminoguanidine, injected at this dose, was shown previously to markedly affect the susceptibility of mice to *Listeria monocytogenes* infection, without toxic effects (4). Since the half-life of aminoguanidine is approximately 14 h (4), BALB/c and DBA/2 mice received this drug twice daily starting 2 days before infection. Treatment with aminoguanidine resulted in significantly higher numbers of *P. aeruginosa* in the lungs of resistant (BALB/c) but not susceptible (DBA/2) mice at 3 days postinfection. These findings are indicative that NO, secreted by either PMN and/or macrophages, could be an important effector molecule involved in the killing of *P. aeruginosa*. The fact that resistant BALB/c mice depleted of NO did not become as susceptible to the infection as DBA/2 mice suggests that NO does not account solely for the genetic difference between BALB/c and DBA/2 in terms of resistance to *P. aeruginosa* infection. Superoxide could be another potential effector molecule also responsible for the killing of *P. aeruginosa*. The study reported by Hostoffer and colleagues (15) indeed suggested that TNF-α regulates the immunoglobulin A-mediated superoxide generation, a function which was associated with an enhanced killing of *P. aeruginosa*.

The mechanisms underlying host defense against murine infection with *P. aeruginosa* remain not fully understood. Several lines of evidence, however, indicate that a rapid and efficient recruitment of PMN and macrophages to the site of infection is crucial for resistance to this infection (27, 29, 40). The involvement of several chemotactic factors such as IL-1, C5, and monocyte-chemotactic and activating factor has been documented in several models of rat and murine infection with *P. aeruginosa* (8, 29, 40). These cytokines as well as other cytokines secreted by $CD4^+$ T lymphocytes (11) were postulated to modulate the bactericidal activity of PMN and/or macrophages, thus underlying the importance of these cell populations in resistance to the infection. In the study reported herein, we have presented another aspect of phagocyte activation for bactericidal activity in host response to lung infection with *P. aeruginosa*. We postulate that TNF-α is implicated in host resistance to pulmonary infection with *P. aeruginosa*, most likely by inducing macrophages and/or PMN to secrete NO, a factor that could be involved directly in the killing of *P. aeruginosa*.

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