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Role of Tumor Necrosis Factor Alpha in Pathogenesis of Pneumococcal Pneumonia in Mice

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The production and role of tumor necrosis factor alpha (TNF-α) in pneumococcal pneumonia were investigated in a mouse pneumonia model. When approximately 10^6 CFU of *Streptococcus pneumoniae* TUM19 were used to inoculate CBA/J mice intranasally, TNF-α levels in the lungs and serum began to increase from 1 and 3 days after infection, respectively, concomitantly with the increase in bacterial counts in the lungs. Anti-TNF-α antibody accelerated bacterial proliferation in the blood and the death of the mice. Although serum levels of immunoglobulin G antibody against the infecting bacteria were not affected by the anti-TNF-α antibody treatment, neutrophil counts in the blood were decreased by the treatment. These results suggest that TNF-α produced in the course of pneumococcal pneumonia prevents bacteremia by increasing the number of neutrophils in the blood.

Streptococcus pneumoniae is one of the major pathogens of community-acquired pneumonia, leading to severe disease (18, 24). Despite the use of potent antibiotics, *S. pneumoniae* continues to cause considerable morbidity and mortality throughout the world. A better understanding of the pathogenesis of pneumococcal pneumonia is needed to control this serious disease.

Pneumococcal pneumonia is characterized by an intense inflammatory reaction which is known to be directly induced by pneumococcal cell wall components and pneumolysin (12). These pneumococcal components and products, on the other hand, have been shown to stimulate production of tumor necrosis factor alpha (TNF-α) by human monocytes in vitro (10, 11). In fact, elevated levels of TNF-α have been observed in the serum and cerebrospinal fluid of patients with acute lower respiratory tract infection and meningitis, respectively, caused by *S. pneumoniae* (8, 17). In addition, it has been demonstrated that TNF-α is an important mediator of inflammation and tissue damage in pneumococcal meningitis (20). However, the role of TNF-α in the pathogenesis of pneumococcal pneumonia is not clear.

We have recently established a mouse pneumonia model with *S. pneumoniae* which closely mimicks the situation in humans (22). By using this model, we investigated endogenous TNF-α production and its role in pneumonia by administration of anti-TNF-α antibody. Our results show that endogenously produced TNF-α plays a protective role in pneumococcal pneumonia.

MATERIALS AND METHODS

Mice. Five- to seven-week-old female CBA/J mice were obtained from Charles River Japan, Inc., Kanagawa.

Organism. A clinical isolate of *S. pneumoniae* serotype 19 (strain TUM19), stocked in the Department of Microbiology, Toho University School of Medicine, Tokyo, Japan, was used in this study. The strain is penicillin resistant, and the MIC of penicillin G against the organism was 2.0 μg/ml. The organisms were incubated at 37°C for 20 h on Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) plates supplemented with 5% defibrinated horse blood and suspended in sterile physiological saline.

Respiratory tract infection. Mice were anesthetized by intraperitoneal injection of 50 mg of sodium pentobarbital (Abbott Laboratories, North Chicago, Ill.) per kg of body weight and challenged intranasally with 40 μl of a bacterial suspension containing approximately 10^6 CFU of *S. pneumoniae* TUM19.

Bacteriological examination. Mice were killed by bleeding from the axillary artery and vein under ether anesthesia. The lungs were removed aseptically and homogenized with 2 ml of sterile physiological saline by using a Teflon tissue homogenizer. The homogenates and blood were serially diluted 10-fold with sterile physiological saline, and 0.1-ml samples of the various dilutions were inoculated onto Mueller-Hinton agar plates supplemented with 5% defibrinated horse blood. The plates were incubated at 37°C for 20 h. Colonies were enumerated, and bacterial counts in the lungs and blood were expressed as the log number of CFU per organ or per milliliter of blood.

Preparation of serum. At various times after infection, blood was obtained from the axillary artery and vein. Individual sera were separated from the clotted blood by centrifugation and stored at −80°C until antibody and TNF-α assays were performed.

Preparation of BAL. At various times after infection, bronchoalveolar lavages (BAL) were obtained by injecting and aspirating 0.4 ml of Hanks' balanced salt solution (Bio Whittaker, Walkersville, Md.). This operation was repeated five times per mouse. The obtained BAL were pooled and stored at −80°C until TNF-α assays were performed.

TNF-α assay. TNF-α concentrations in the serum and BAL of infected mice were measured with a Mouse TNF-α ELISA kit (Genzyme, Cambridge, Mass.) as described in the manufacturer's instructions. Briefly, samples were incubated with a hamster monoclonal antibody for murine TNF-α adsorbed onto the wells. The captured TNF-α was detected with a goat polyclonal anti-murine TNF-α antibody and a horseradish peroxidase-conjugated anti-goat immunoglobulin. The enzyme substrate was *o*-phenylenediamine, and the optical density at 450 nm was read in a spectrophotometer (Multiskan Bichromatic; Labsystems). Sample concentrations were read from a dose-response curve obtained with reference recombinant TNF-α (detection limit, 15 pg/ml).

Antibody assay. Serum levels of immunoglobulin G (IgG) antibody against the infecting organisms were measured with a Microwell ELISAmate kit (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) as described in the manufacturer's instructions. Enzyme-linked immunosorbent assay plates (ICN Biomedicals, Inc., Horsham, Pa.) were coated with heat-killed *S. pneumoniae* TUM19 suspended in coating solution at a concentration of approximately 10^9 cells/ml and incubated overnight at 4°C. Coated plates were incubated with the serum samples and a phosphatase-labeled, affinity-purified goat anti-mouse IgG antibody (γ chain specific; Kirkegaard & Perry Laboratories). The enzyme substrate was *p*-nitrophenylphosphate in diethanolamine buffer, and the optical density at 405 nm was read in a spectrophotometer (Labsystems).

Blood leukocyte assay. Blood samples were obtained from the axillary artery and vein. Cell number was counted with a hemacytometer. Differential counts were performed by Giemsa staining of monolayers.

In vivo neutralization of TNF-α. Mice were injected intravenously with 10^4 U of polyclonal rabbit anti-mouse TNF-α antibody (neutralizing activity, 10^5 U/ml; Genzyme) or normal rabbit serum 1 and 4 days after infection. It is known that

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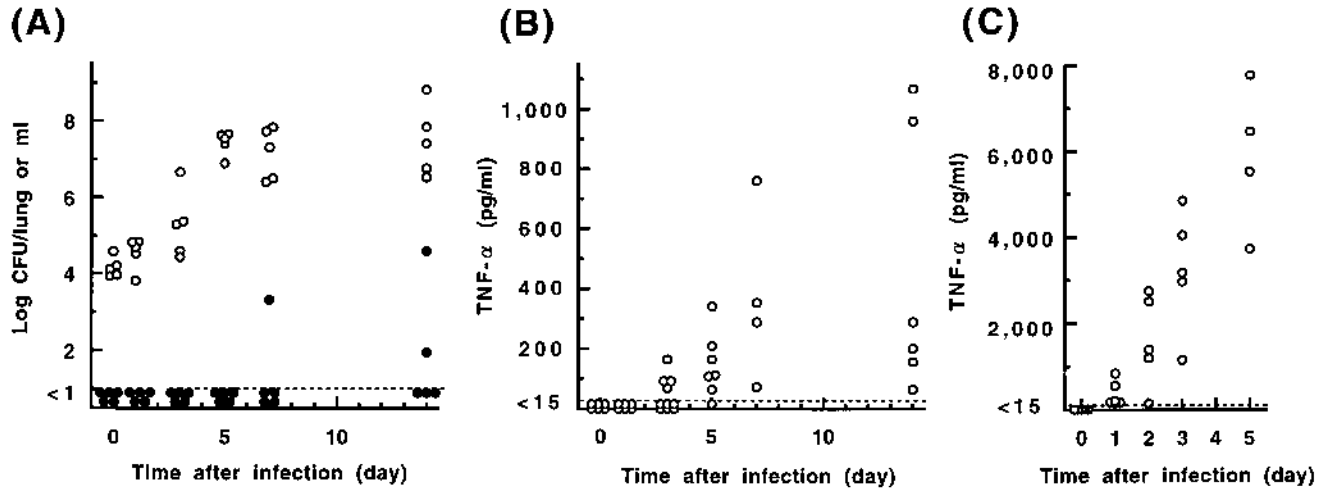

FIG. 1. Bacterial counts in the lungs (○) and blood (●) (A), TNF-α levels in the serum (B), and TNF-α levels in the lungs (C) of mice infected with *S. pneumoniae* TUM19 by intranasal instillation. Each point represents the value for a mouse.

4×10^5 U of this anti-TNF-α antibody is sufficient to neutralize up to 100 ng of TNF-α per milliliter of blood of lipopolysaccharide-treated mice in vivo (5). The number of survivors was recorded daily. Surviving mice were sacrificed 7 days after infection and examined for bacterial counts in the lungs and blood, levels in serum of IgG antibody against the infecting bacteria, and cell counts in blood.

Statistical analysis. Differences between normal rabbit serum-treated and anti-TNF-α antibody-treated groups in bacterial counts in the lungs and blood, levels in serum of IgG antibody against the infecting bacteria, and cell counts in blood were analyzed with the Dunnet type test. Differences in survival rate were analyzed with Fisher's exact test.

RESULTS

Production of TNF-α in mice with pneumococcal pneumonia. After intranasal instillation of approximately 10^6 CFU of *S. pneumoniae* TUM19, the bacterial counts in the lungs increased slowly during the first 3 days after infection and then increased rapidly, reaching 10^7 CFU at 5 days (Fig. 1A). The number of bacteria in the lungs increased gradually thereafter. Some mice developed bacteremia when the bacterial counts in the lungs reached more than 10^8 CFU from 7 days after infection. In parallel, the concentrations of TNF-α in the sera and lungs of infected mice were measured. TNF-α was first detected in serum 3 days after infection, and its levels increased thereafter. Serum TNF-α levels at 14 days after infection were distributed over a wide range of 100 to 1,000 pg/ml (Fig. 1B). Three mice that developed bacteremia 7 and 14 days after infection showed high TNF-α levels (more than 800 pg/ml) in serum. In contrast, TNF-α was first detected in the lungs 1 day after infection and its levels increased rapidly, reaching approximately 6,000 pg/ml at 5 days (Fig. 1C).

Effect of anti-TNF-α antibody. Mice were injected intravenously with 10^4 U of anti-TNF-α antibody or normal rabbit serum 1 and 4 days after infection. Mice that received anti-TNF-α antibody did not produce detectable TNF-α levels (<15 pg/ml) in serum 7 days after infection (data not shown). We could not obtain adequate BAL 7 days after infection because solid lobar consolidation and severe hemorrhage existed in the lungs (22). As far as we examined TNF-α levels in BAL under these conditions, TNF-α was not detected (<15 pg/ml) in BAL obtained from anti-TNF-α antibody-treated mice, whereas approximately 2,500 pg of TNF-α per ml was detected in BAL from normal rabbit serum-treated mice. The survival rate of each group was observed for 14 days (Fig. 2). Five of 15 normal rabbit serum-treated mice died from 8 to 14 days after infection, whereas 11 of 15 anti-TNF-α antibody-treated mice died 6 to 14 days after infection. The mortality recorded on days 9, 12, and 13 was significantly higher in anti-TNF-α antibody-treated mice than in normal rabbit serum-treated mice ($P < 0.05$).

Next, the effect of the anti-TNF-α antibody on bacterial counts in the lungs and blood 7 days after infection was examined (Fig. 3A). Anti-TNF-α antibody-treated mice had almost the same number of bacteria in the lungs as did normal rabbit serum-treated mice. However, the bacterial counts in the blood of mice which received anti-TNF-α antibody were significantly higher than those of normal rabbit serum-treated mice ($P < 0.01$).

Efficient opsonization of bacteria by type-specific antibody and complement for phagocytosis and killing by phagocytes is important for the removal of pneumococci from the lungs and blood (3, 9). Titers of specific IgG antibody against the infect-

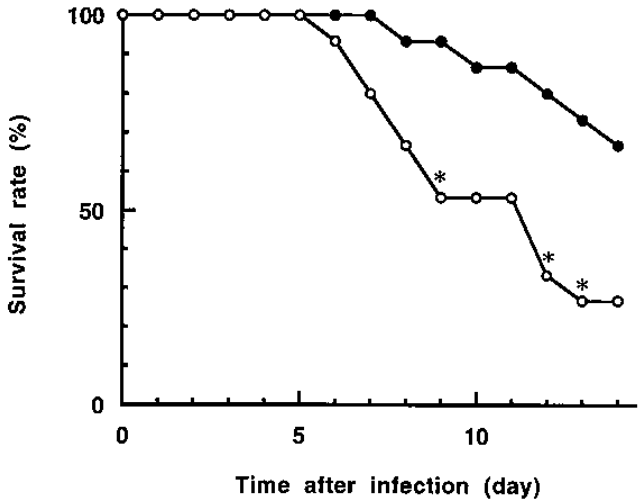

FIG. 2. Effect of anti-TNF-α antibody on survival rates of mice infected with *S. pneumoniae* TUM19 by intranasal instillation. Each of 15 mice per group was injected intravenously with 10^4 U of anti-TNF-α antibody (○) or normal rabbit serum (●) 1 and 4 days after infection. *, $P < 0.05$ compared with values for normal rabbit serum-treated mice.

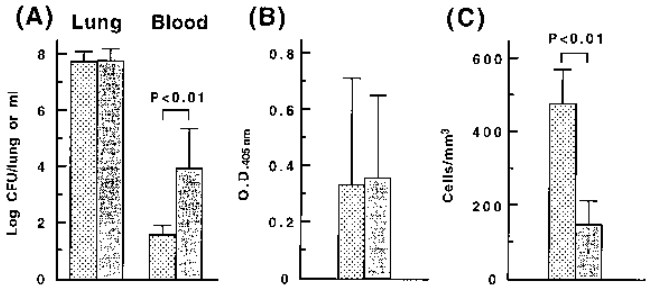

FIG. 3. Effect of anti-TNF-α antibody on bacterial counts in the lungs and blood (A), serum levels of IgG antibody against the infecting bacteria (B), and neutrophil counts in the blood (C) of mice infected with *S. pneumoniae* TUM19 by intranasal instillation. Each of 3 to 11 mice per group was injected intravenously with 10^4 U of anti-TNF-α antibody (■) or normal rabbit serum (▨) 1 and 4 days after infection. Error bars indicate the standard deviation of the mean. $P < 0.01$ compared with values for normal rabbit serum-treated mice.

ing bacteria were elevated from 5 days after infection in this model (data not shown). In addition, it is known that TNF-α stimulates B cells and accelerates antibody production (13, 19). Therefore, we examined the effect of endogenously produced TNF-α on antibody production by comparing the serum titers of IgG antibody against the infecting bacteria in anti-TNF-α antibody-treated and normal rabbit serum-treated mice 7 days after infection (Fig. 3B). There was no significant difference in serum titers of IgG antibody between the treatment groups.

It is also known that TNF-α has various biological effects on neutrophils (7, 14–16, 21, 23), which play an important role in the killing of pneumococci (4). Therefore, we next examined the effect of anti-TNF-α antibody on the number of blood neutrophils (Fig. 3C). Anti-TNF-α antibody-treated mice had significantly smaller numbers of neutrophils in blood 7 days after infection than did normal rabbit serum-treated mice ($P < 0.01$).

DISCUSSION

TNF-α has been detected in cerebrospinal fluid of patients with pneumococcal meningitis (8) and rabbits with experimental pneumococcal meningitis (6), and it has been reported that TNF-α has multiple inflammatory activities in the central nervous system and causes tissue damage (20). Concerning pneumococcal pneumonia, Nohynek et al. (17) have reported that the serum level of TNF-α was elevated in patients with acute lower respiratory tract infection. However, there have been no studies on the role of TNF-α in pneumococcal pneumonia.

In the present study, we showed that TNF-α was produced in the serum and lungs of mice with pneumococcal pneumonia in parallel with bacterial growth in the lungs. The TNF-α response in the lungs rose more rapidly than the serum TNF-α response, and TNF-α levels in the lungs were much higher than TNF-α levels in serum. These results might better reflect a local infection. In addition, we found that the infection was worsened by administration of anti-TNF-α antibody. Treatment with anti-TNF-α antibody increased the number of infecting bacteria in the blood and accelerated the death of infected mice. These results suggest that TNF-α prevents the onset of bacteremia. It is well known that major mechanisms protecting the host from pneumococcal infections are opsonization of organisms by type-specific antibody and complement and phagocytosis and killing of opsonized bacteria by macrophages and neutrophils (3, 9). Since it is also known that TNF-α stimulates antibody production (13, 19) and activates neutrophils (7, 14–16, 21, 23), we considered that the antibody production and/or neutrophil activity might be suppressed by neutralization of endogenously produced TNF-α. Although the levels of IgG antibody in serum were not affected by anti-TNF-α antibody treatment, neutrophil counts in the blood were reduced by the treatment. Accordingly, it is likely that the bacterial proliferation in the blood of anti-TNF-α antibody-treated mice was caused by the decrease of neutrophil counts in the blood. Azoulay-Dupuis et al. (1) have shown that mice with neutropenia induced by cyclophosphamide treatment developed acute pneumonia with bacteremia after intratracheal injection of pneumococci. Therefore, it is believed that neutrophil counts are critical in protection against pneumococcal pneumonia. The results of this study suggest that endogenously produced TNF-α increases neutrophils in blood and indirectly affords protection against invasion of the circulatory system by infecting bacteria in the lungs in pneumococcal pneumonia. It should be pointed out that we have only examined the neutrophil response systemically. We think that the local phenomena in the lungs should be clarified, since a neutrophil response must occur in the lungs, where persistent stimulation by the infecting bacteria exists. BAL has often been performed to examine the cellular response in the lungs. However, it was difficult to obtain adequate information directly from lung washings of infected mice because solid lobar consolidation formed and severe hemorrhage was seen in the lungs of infected mice after 5 days of infection. Therefore, we cannot adequately discuss the neutrophil response in the lungs. Bacterial counts in the lungs of anti-TNF-α antibody-treated mice showed no marked changes 7 days after infection, whereas those in the blood of anti-TNF-α antibody-treated mice significantly increased. Accordingly, it is possible that the decrease of circulating neutrophils caused by anti-TNF-α antibody treatment might make an important contribution to bacterial proliferation in the blood and death of the infected mice than the change of neutrophils in the lungs. TNF-α is known to stimulate granulocyte-macrophage colony-stimulating factor production by human endothelial cells (2). Therefore, it is thought that TNF-α stimulates granulocyte-macrophage colony-stimulating factor production, indirectly enhances the recruitment of neutrophils, and indirectly affords protection against invasion of the circulatory system by infecting bacteria in the lungs.

We demonstrated that TNF-α plays a protective role in experimental pneumococcal pneumonia by suppressing the occurrence of bacteremia. In addition to TNF-α, pneumococcal cell wall components and pneumolysin are known to stimulate the production of interleukin-6 and interleukin-1β, respectively, by human monocytes in vitro (10, 11). These cytokines also may play important roles in pneumococcal pneumonia.

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