Tumor Necrosis Factor Alpha and Gamma Interferon are Required for the Development of Protective Immunity to Secondary Corynebacterium pseudotuberculosis Infection in Mice

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(Received 26 April 1999/Accepted 13 July 1999)

ABSTRACT. The production and role of endogenous cytokines during the course of secondary Corynebacterium (C.) pseudotuberculosis infection were investigated in mice. When immunized mice were challenged on day 28 after primary infection, tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) were found to appear at 3 hr and to reach the maximum at 24 hr after challenge. Spleen cells of mice primarily infected from 2 to 8 weeks before produced a significant amount of TNF-α and IFN-γ when stimulated with formalin-killed bacteria. However, they could not produce detectable amounts of IL-4. The administration of anti-TNF-α monoclonal antibody (MAb) and IFN-γ MAb increased bacterial proliferation in the organs of immune mice and exacerbated the secondary infection. Injection of anti-CD4 MAb alone or anti-CD4 plus anti-CD8 MAb s resulted in significantly increased mortality and a marked suppression of bacterial elimination as well as cytokine production of secondarily infected mice, while the treatment with anti-CD8 MAb alone showed no effect on either the resistance or cytokine production of mice. These results suggest that CD4, probably Th1 T cells, play an important role for establishment of protective immunity against secondary C. pseudotuberculosis infection by secreting TNF-α and IFN-γ—key words: Corynebacterium pseudotuberculosis, IFN-γ secondary infection, TNF-α.

Corynebacterium (C.) pseudotuberculosis is a facultative intracellular pathogen that causes caseous lymphadenitis (CLA), a worldwide disease in both sheep and goats [4, 5, 25]. CLA is characterized by the development of pyogranulomas in lymph nodes and lungs [3]. The immune response to C. pseudotuberculosis is complex and includes both humoral and cellular mechanisms [6, 7, 11–13, 16]. T lymphocytes as well as macrophages are reportedly play an important role in host defense against C. pseudotuberculosis [13, 14]. Recent studies have revealed the complexity of the cytokine and cellular interactions required for resistance to primary C. pseudotuberculosis infection and have illustrated that resistance to secondary infection may occur through multiple pathway [21]. TNF-α has been recognized as an important factor in immunity to C. pseudotuberculosis [9, 10]. The role of IFN-γ in host resistance was examined in knock-out mice deficient for the IFN-γ receptor, and the result demonstrated that IFN-γ is needed to overcome a primary C. pseudotuberculosis infection [23]. Our previous studies [17] revealed that TNF-α and IFN-γ are both essential for the development of host defense against primary infection with C. pseudotuberculosis. However, the role of these cytokines during a secondary C. pseudotuberculosis infection has not yet been studied. Therefore, the present study was undertaken to examine the requirement of TNF-α and IFN-γ for the expression of protective immunity against C. pseudotuberculosis during a secondary infection in mice.

MATERIALS AND METHODS

Mice: Female 6- to 8-week-old ICR-JCL mice were purchased from a commercial breeder (CLEA Japan Inc., Tokyo) and maintained in our laboratory under conventional conditions throughout the experiment.

Bacteria: C. pseudotuberculosis ATCC 19410 strain was grown in a brain-heart infusion broth containing 0.1% Tween 80 (BHI-T) for 48 hr at 37°C and frozen at -80°C until use. The 50% lethal dose (LD50) of this strain was approximately 7.9 × 10^6 colony forming unit (CFU) for normal mice.

Induction of secondary C. pseudotuberculosis infection: A secondary infection was induced in mice that had survived the primary intraperitoneal (i.p.) injection with 2 × 10^6 C. pseudotuberculosis (0.25 LD50) by intravenously (i.v.) injecting 20 LD50 C. pseudotuberculosis on different days after the primary injection.

Monoclonal antibodies: The following MAb s were used: anti-mouse TNF-α (MP6-XT22.11; rat IgG1), anti-mouse IFN-γ (R4-6A2; rat IgG1), anti-CD4 (GK1.5, rat IgG2b), and anti-CD8 (53-6.72, rat IgG2a). These MAb s were produced by hybridomas growing as ascites fluids in pristane-primed KSN nude mice. Partial purification by 50% (NH4)2SO4 precipitation was followed by exhaustive dialysis against PBS as described previously [17].

In vivo depletion of endogenous cytokines: Each mouse

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was given a single i.p. injection of 2 mg of anti-TNF-α MAb or/and anti-IFN-γ MAb 2 hr before secondary infection. Normal rat globulin (NRaG) was injected as a control for the Mabs. NRaG was prepared as described previously [20].

In vivo depletion of CD4+ and CD8+ cells: Each mouse was given a single i.p. injection of 2 mg of anti-CD4 MAb or anti-CD8 MAb on day 1 before the secondary infection. NRaG was injected as a control.

Preparation of formalin-killed bacteria: C. pseudotuberculosis was grown in BHI-T for 48 hr at 37°C. The bacterial cells were harvested, washed three time with 0.01 M phosphate-buffered saline (PBS, pH 7.2) by centrifugation at 2,000 × g for 30 min, and suspended in 1% formalin in PBS for 3 days at 4°C. After that, the killed bacteria were harvested, washed 3 time with PBS, and resuspended in PBS at the desired concentration (wet weight).

Cell culture: Spleens were aseptically removed from infected mice and minced into single cells. After lysing red blood cells with 0.83% ammonium chloride solution (pH 7.4), spleen cells were washed three times with Hank’s balanced salt solution and suspended at a concentration of 106 cells/ml in culture medium consisting of RPMI 1640 supplemented with 10% fetal calf serum (FCS, Flow Australia), penicillin (100 units/ml), and streptomycin (100 µg/ml). One ml of cell suspensions were seeded in each well of 24-well microplates. Suspensions of formalin-killed bacteria were added at a final concentration of 50 µg/ml and incubated for 72 hr in an atmosphere of 5% CO2, 95% air at 37°C. Culture supernatants were collected by centrifugation at 1,000 × g for 20 min and stored at -80°C.

Preparation of samples for cytokine assay: At different times after secondary infection, the serum and spleen samples for TNF-α and IFN-γ assay were prepared as described previously [17]. Briefly, spleens were aseptically removed from the mice and suspended in RPMI-1640 medium containing 1% (wt/vol) 3-((cholamidopropyl)dimethyl-ammonio)-1-propanesulfonate (CHAPS; Wako Pure Chemical Co., Osaka, Japan), and 10% (wt/vol) homogenates were prepared with a glass homogenizer (U.S. type, Ikemoto Riko Co., Ltd., Tokyo). After the homogenates were left for 1 hr on ice, they were clarified by centrifuging at 1,500 × g for 20 min. The organ extracts were stored at -80°C until assayed for cytokine activities.

Assay for cytokines: The TNF-α and IFN-γ assays were carried out by an enzyme-linked immunosorbent assay (ELISA) as described previously [17]. The IL-4 activity was measured using a Cytoscreen™ mouse IL-4 immunoassay kit (Lot #101395, Camarillo, CA, U.S.A.), as described in the manufacturer’s instructions.

Determination of numbers of viable C. pseudotuberculosis cells in organs: The numbers of viable C. pseudotuberculosis cells in the organs of infected mice were established by plating serial 10-fold dilutions of organ homogenates in PBS on BHI agar. Colonies were routinely counted 48 hr later.

Statistical analysis: Data were expressed as mean ± standard deviation. The statistical significance for the comparison of cytokine titers between groups was determined by Student’s t-test. The chi-square test was used to determine the significance of the survival rate. A p value of less than 0.05 was considered statistically significant.

RESULTS

Appearance and persistence of primary infection-induced resistance to secondary infection: At different times after primary i.p. infection mice were secondarily infected with 20 LD50 of C. pseudotuberculosis and survival was observed for 30 days. Non-immunized mice were used for control. As can be seen in Fig. 1, while all control mice died within 6 days, 50, 70, 90, 100 and 90% of mice that were challenged 2, 3, 4, 5 or 8 weeks after primary infection survived the lethal challenge, respectively. A significantly enhanced survival rate was observed if mice were challenged at the time between 2 and 8 weeks after primary infection.

In vitro production of TNF-α, IFN-γ and IL-4 by spleen cells: To investigate the profile of in vitro cytokine production, infected mice spleen cells were harvested at different times after primary i.p. infection, and were incubated with formalin-killed bacteria. Spleen cells that harvested from 2 to 8 weeks after primary infection produced a significant amount of TNF-α and IFN-γ, but they could not produce significant amounts of IL-4. No detectable amount of cytokine was produced by spleen cells of uninfected control mice even though they were stimulated with formalin-killed bacteria. Spleen cells from infected mice also could not produce any cytokine if they were not stimulated with formalin-killed bacteria (Table 1).

Kinetics of bacterial growth and endogenous cytokine production during secondary infection: To investigate the
correlation between the kinetics of bacterial proliferation and cytokine production, immunized mice that had recovered from a primary infection were challenged with 20 LD50 of *C. pseudotuberculosis* 4 weeks later. The bacteria proliferated in the spleens and livers only during the first day and were eliminated rapidly from these organs during the next 2 days of secondary infection (Fig. 2A). In parallel, the titers of IFN-γ, TNF-α and IL-4 were determined in the sera and spleen homogenates. IFN-γ titers began to increase in the sera as well as in the spleen samples 3 hr after reinfection, peaked at 24 hr, and then decreased. No IFN-γ was detected in the sera after day 4, but was still detected in the spleen homogenates until day 10 after challenge (Fig. 2B). In the spleens, TNF-α appeared at 3 hr, peaked at 24 hr, and thereafter decreased gradually, but no TNF-α was detectable in any of the serum samples (Fig. 2C). IL-4 was undetectable in both serum and spleen samples (data not shown).

**Effect of anti-TNF-α and anti-IFN-γ MAbs on survival rates:** Immunized mice were injected i.p. with anti-TNF-α MAb or anti-IFN-γ MAb or both 2 hr prior to challenge. The survival of each group was observed for 30 days. As shown in Fig. 3, every control nonimmunized mouse died within 6 days of the challenge, and 50% and 60% of anti-IFN-γ MAb- and anti-TNF-α MAb-treated mice died from infection, respectively, whereas the immunized controls that had received NRaG survived the lethal bacterial challenge. The mortality of mice that received both anti-TNF-α and anti-IFN-γ MAbs was the same as that in mice treated with anti-TNF-α MAb alone. None of the immunized mice that treated with anti-TNF-α MAb or anti-IFN-γ MAb but not challenged died (data not shown).

**Effect of anti-TNF-α and anti-IFN-γ MAbs on bacterial growth:** Immunized mice were injected i.p with anti-TNF-α MAb, anti-IFN-γ MAb or both 2 hr before secondary infection. Two days later, the numbers of bacterial cells in the spleens and livers were determined. After challenge, elimination of bacteria from the spleens and livers of immunized mice was significantly blocked in both anti-TNF-α MAb- and anti-IFN-γ MAb-treated mice, as compared with that in NRaG-treated control mice (Fig. 4). However,
the number of bacteria in both organs from mice which had received anti-TNF-α MAb was higher than in organs from anti-IFN-γ MAb-treated mice. The bacterial number in the spleens of immunized mice treated with either anti-TNF-α MAb or anti-IFN-γ MAb but not challenged was at undetectable level on days 28 and 31 of primary infection (data not shown).

**Effect of administration of anti CD4 and anti CD8 on resistance and cytokine production:** To investigate the role of T cell subsets on the development of protective immunity during a secondary infection, immunized mice were injected with anti-CD4 MAb, anti-CD8 MAb or both 1 day prior to challenge. Fifty percent of anti-CD4 MAb-treated mice and 50% of mice receiving both anti-CD4 and anti-CD8 MAbs died from infection, even though they had been immunized (Table 2). After secondary infection, an explosive multiplication of bacteria was observed in the organs from mice that received anti-CD4 MAb alone or simultaneous administration of both MAbs. None of the immunized mice died when treated with anti-CD4 MAb but not challenged (data not shown). Administration of anti-CD8 MAb did not enhance the mortality of infected mice nor significantly suppress the elimination of bacteria from the organs (p>0.05). The treatment with anti-CD4 MAb alone or anti-CD4 plus anti-CD8 MAbs also significantly inhibited the production of both IFN-γ and TNF-α, while anti-CD8 MAb alone showed no effect on the production of these cytokines.

**DISCUSSION**

Although both TNF-α and IFN-γ are reportedly essential for the development of host defense against primary *C. pseudotuberculosis* infection in mice [17], the role of these cytokines during a secondary infection has not yet been clarified. Therefore, the present study was undertaken to examine the role of TNF-α and IFN-γ in protective immunity during a secondary *C. pseudotuberculosis* infection. The observation that 50% of mice infected 2 weeks before could survive a lethal challenge injection (Fig. 1) suggests that after primary infection an acquired resistance to the bacterium can be developed after 2 weeks of primary infection and retained for at least 6 weeks thereafter. Spleen cells that harvested from 2 to 8 weeks after primary infection produced a significant amount of TNF-α and IFN-γ when stimulated with formalin-killed bacteria, suggesting that the establishment of host resistance...
induced by primary infection correlates with the production of these cytokines.

In the primary infection, the bacteria proliferated during the first 3 days and were eliminated from the organs during the next several days [17], whereas in the secondary infection, bacterial proliferation in the organs occurred only during the first 24 hr, and elimination of bacteria took place during the next 2 days (Fig. 2A). Thus, mice with a secondary *C. pseudotuberculosis* infection eliminate the bacteria much faster and more efficiently from their organs than mice with a primary infection. This is in agreement with previous studies reporting that in convalescent mice, multiplication of *C. pseudotuberculosis* was more restricted and the organism eliminated more quickly than in normal controls [16]. This elimination may be the result of a quick and augmented response of established immunity including activated macrophages [15]. Therefore, it is likely that the cellular responses inducing cytokine production would occur rapidly and intensively in a secondary infection. In fact, we were able to detect significant TNF-α and IFN-γ activities in immunized mice soon after a secondary challenge with *C. pseudotuberculosis*. Both TNF-α and IFN-γ appeared in mice within 3 hr and reached the maximum at 24 hr. During the course of secondary infection (Figs. 2 B, C), the amounts of IFN-γ and TNF-α were higher than those during a primary infection [17].

The treatment of anti-TNF-α and anti-IFN-γ MAbs increased mortality and bacterial growth in the organs of mice (Figs. 3, 4). This suggests that endogenous TNF-α and IFN-γ produced in mice during the secondary infection play an important role in the host resistance to *C. pseudotuberculosis*. These cytokines also reportedly play a protective role in host resistance to secondary infection caused by other facultative intracellular pathogens such as *Listeria monocytogenes* [19, 22], *Mycobacterium avium* [2], *Francisella tularensis* [24], *Leishmania major* [18] and *Histoplasma capsulatum* [1].

The precise mechanism by which TNF-α and IFN-γ induce enhanced resistance during a secondary infection is unknown. Possibly, these cytokines may participate in the establishment of protective immunity through activation of macrophages, since their capacity to activate the bactericidal function of macrophages is well established [8, 19]. Jolly [16] reported that macrophages from the peritoneal cavity of infected mice clearly had an enhanced ability to inhibit multiplication or kill *C. pseudotuberculosis* within their cytoplasm. Hard [11] also showed that the acquired immunity appeared to be associated with the resident macrophages of the immunized peritoneal cavity, which were increased in both numbers and size and had altered staining properties. Furthermore, in our experiments, the administration of carrageenan as well as the treatment of anti CD11b MAb (5C6), an MAb effectively inhibits myelomonocytic accumulation at inflammatory foci, resulted in a rapid death of immunized mice after challenge, suggesting the important role of macrophages in immunity to *C. pseudotuberculosis* (manuscript is being processed).

The administration of anti-CD4 MAb significantly increased mortality as well as bacterial proliferation and markedly reduced the production of IFN-γ and TNF-α. These results suggest that CD4 T cells are involved in acquired resistance to secondary *C. pseudotuberculosis*. Therefore, it may be assumed that armed macrophages that activated by TNF-α and IFN-γ produced by sensitized CD4 T cells play a critical role for the elimination of bacteria.

In contrast to the effect of anti CD4 MAb, anti-CD8 MAb did not enhance mortality and showed no significant effect on either elimination of bacteria from the organs or on cytokine production (Table 2). Our previous data have shown that both CD4 and CD8 T cells play an essential role in the establishment of protective immunity against a primary infection caused by *C. pseudotuberculosis* [17]. Thus, the role of CD8 T cells in the development of protective immunity against secondary infection was

### Table 2. Effect of administration of anti-CD4 and anti-CD8 MAbs on resistance and cytokine production in mice during secondary infection with *C. pseudotuberculosis*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bacteria (Log CFU/organ)</th>
<th>Mortality (%)</th>
<th>TNF-α (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spleen</td>
<td>Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NRaG</td>
<td>3.76</td>
<td>3.92</td>
<td>0</td>
<td>1620 ± 237</td>
</tr>
<tr>
<td>Anti-CD8</td>
<td>4.25</td>
<td>4.32</td>
<td>10</td>
<td>1428 ± 190</td>
</tr>
<tr>
<td>Anti-CD4</td>
<td>5.01**</td>
<td>5.28**</td>
<td>50</td>
<td>1256 ± 108*</td>
</tr>
<tr>
<td>Anti-CD4 plus anti-CD8</td>
<td>5.15**</td>
<td>5.32**</td>
<td>50</td>
<td>1206 ± 155*</td>
</tr>
</tbody>
</table>

a) Immune mice received 2 mg of anti-CD4 or anti-CD8 mAb or both on day -1 of secondary infection.
b) Bacterial numbers (mean ± S.D.) from 5 mice were determined on day 2 after secondary infection.
c) Mortality was determined on day 30 from 10 mice.
d) Titers (mean ± S.D.) in spleen extracts from 5 mice were determined on day 1 after secondary infection.

* p<0.05; ** p<0.01.
different from that against primary infection.

Our results showed that IFN-γ and TNF-α are produced rapidly after secondary infection as well as by spleen cells of infected mice upon stimulation with killed C. pseudotuberculosis while IL-4 was undetectable (Fig. 2 and Table 1), suggesting that Th1 cells seem to be important in the host immune response against secondary C. pseudotuberculosis infection.

REFERENCES


