

Role of Gamma Interferon and Tumor Necrosis Factor Alpha in Resistance to *Salmonella typhimurium* Infection

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In mice infected with a sublethal dose of *Salmonella typhimurium*, the injection of an anti-gamma interferon (IFN- γ) monoclonal antibody increased bacterial proliferation in the spleen and led to death on day 7 or 8. Depletion of both CD4⁺ and CD8⁺ T cells with monoclonal antibodies in vivo had a much less marked effect during the first week of infection than the administration of anti-IFN- γ antibodies, suggesting that cells other than T lymphocytes participate in the production of IFN- γ at this time. Administration of anti-tumor necrosis factor alpha (TNF- α) antibodies to mice infected with a sublethal dose of *S. typhimurium* induced the same effect as anti-IFN- γ antibodies, while the administration of both antibodies resulted in a synergistic interaction. When mice were infected with an avirulent strain of *S. typhimurium* and challenged on day 7 either with a virulent strain of *S. typhimurium* or with *Listeria monocytogenes*, their resistance to reinfection was slightly depressed by anti-IFN- γ or anti-TNF- α antibodies given 1 day before challenge and much more strongly depressed by the simultaneous administration of both antibodies. Taken together, these results indicate that IFN- γ and TNF- α play an essential role in acquired resistance during the early phase of *S. typhimurium* infection.

Although it is generally assumed that *Salmonella typhimurium* is a facultative intracellular pathogen and that acquired resistance against this bacterium is mainly cell mediated (8), there remains a degree of controversy on both these points (11, 18). After the intravenous (i.v.) inoculation of mice with a sublethal dose, the time course of infection can be divided into three main phases. During the first, the bacteria grow exponentially in the spleen and liver, their net growth rate being regulated by several genes (32), mainly *Ity* (4). After a week, growth is inhibited and the second phase is characterized by a plateau, also lasting about a week. During the third phase the number of bacteria falls progressively in both organs.

Previous studies have shown that the clearance of bacteria during the third phase is regulated by the *H-2* complex (15, 30) and mediated by CD4⁺ T cells (29). Acquired resistance to infection is, however, present at a very early stage. This resistance is reflected (i) by the growth inhibition of the primary inoculum which occurs at the end of the first week (16) and (ii) by an increased resistance to reinfection by *S. typhimurium* and other intracellular pathogens such as *Listeria monocytogenes* (10, 31). T-cell depletion with anti-CD4 or anti-CD8 monoclonal antibodies (MAb) in vivo does not diminish the resistance to infection during the first 2 weeks (29). The mechanism of resistance during this period is still unclear but seems to be T cell independent (16, 20, 29). Studies on *L. monocytogenes* infection show that gamma interferon (IFN- γ) (6) and tumor necrosis factor alpha (TNF- α) (13) play an important role in the early phase of infection. Both of these cytokines can stimulate the bactericidal activity of macrophages (5, 9, 28). The present study shows that these cytokines are also of critical importance in the early phase of *S. typhimurium* infection.

MATERIALS AND METHODS

Mice. Six- to 8-week-old female CBA mice purchased from Iffa Credo (L'Arbresle, France) were used.

Bacteria. Virulent *S. typhimurium* C5; its temperature-sensitive avirulent mutant, C5TS (17); and a virulent strain of *L. monocytogenes* were stored at -80°C . For each experiment a sample was thawed and grown overnight in tryptic soy broth at 37°C (or 30°C for C5TS). Mice were inoculated i.v. with 0.2 ml of an appropriate dilution in saline. The density of viable organisms was determined by plating onto tryptic soy agar.

Enumeration of bacteria in the spleens. Groups of 3 or 4 mice were killed at various intervals. Spleens were homogenized in 2 ml of distilled water. Samples of the homogenates and 10-fold serial dilutions in saline were plated onto tryptic soy agar. Colonies were counted after overnight incubation at 37°C .

Anti-murine IFN- γ MAb. Hybridoma HB170 (33) producing rat immunoglobulin G1 (IgG1) against murine IFN- γ was kindly provided by G. Milon (Institut Pasteur, Paris, France). The hybridoma was grown intraperitoneally in pristane-primed Swiss nude mice (Iffa Credo). Antibodies were partially purified from ascites by 50% ammonium sulfate precipitation. The preparation contained 17 mg of protein per ml and had a neutralizing titer of 1:10,000 against 10 U of recombinant mouse IFN- γ (Holland Biotechnology bv, Leiden, The Netherlands) per ml. IFN- γ activity was measured in terms of protection of L-929 cells from the cytopathic effect of vesicular stomatitis virus. Ascites containing a MAb against an irrelevant antigen (penicilloyl group) was used as a control.

Anti-murine TNF- α antibody. A rabbit was immunized with 10 μg of recombinant murine TNF- α (Genzyme, Boston, Mass.) in Freund's complete adjuvant and boosted 1 and 2 months later with the same amount of recombinant TNF- α in incomplete adjuvant. The rabbit was bled 1 week after each boost. The serum was precipitated with 33% ammonium sulfate, resuspended in a volume of phosphate-buffered saline half that of the original serum sample, and

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dialyzed against phosphate-buffered saline. One milliliter of this preparation neutralized 10^5 U of murine TNF- α . TNF- α activity was assayed as described by Fish and Gifford (12). Briefly, L-929 cells treated with 1 μ g of actinomycin D (Sigma Chimie, La Verpilliere, France) per ml were incubated overnight with 10 U of TNF- α per ml and various amounts of anti-TNF- α serum. The cells were washed with Hanks' buffered saline solution and stained with crystal violet, and optical density was measured at 545 nm. Normal rabbit serum precipitated with ammonium sulfate was used as a control.

T-cell subset depletion. Hybridomas GK1.5, producing rat anti-CD4 IgG2b MAb, and H35-17.2, producing rat anti-CD8 IgG2b MAb (kindly provided by G. Milon [Institut Pasteur]), were grown intraperitoneally in pristane-primed Swiss nude mice. Antibodies were partially purified from ascites by 50% ammonium sulfate precipitation. After dialysis against phosphate-buffered saline, the rat IgG concentration was determined by means of radial immunodiffusion (Serotec, Bicester, United Kingdom). C5-infected CBA mice were injected intraperitoneally on day 0 with 2 mg of anti-CD4 MAb or i.v. with 400 μ g of anti-CD8 MAb. Control mice received 0.2 ml of ascites containing an irrelevant MAb.

Flow cytometric analysis. Cell suspensions were prepared from spleens. Erythrocytes were lysed with Tris-buffered NH_4Cl (pH 7.2) and washed in Hanks' buffered saline solution. Samples of 2×10^6 splenic cells were resuspended in Hanks' buffered saline solution containing 5% fetal calf serum and 0.2% sodium azide. The cells were treated successively, in the cold, with appropriate dilutions of GK1.5 MAb and fluorescein isothiocyanate-labeled mouse anti-rat κ -chain antibody (Mark-1; Biosys, Compiègne, France) for the detection of CD4 $^+$ T cells or with biotinylated anti-Lyt-2 antibody and fluorescein isothiocyanate-labeled avidin (both from Becton Dickinson, Mountain View, Calif.) for the detection of CD8 $^+$ T cells. After washing, the cells were analyzed in a flow cytometer (Facsan; Becton Dickinson) gated to exclude nonviable cells.

Statistical analysis. Student's unpaired *t* test was used to determine the significance of the differences between control and experimental groups.

RESULTS

Effects of anti-IFN- γ MAb on the early phase of *S. typhimurium* infection. Naturally resistant CBA mice were infected i.v. with a sublethal dose (10^3 CFU) of *S. typhimurium* C5 and treated with 400 μ g of anti-IFN- γ or control MAb. Spleen counts increased progressively in control mice until day 7 and then reached a plateau. In mice treated with anti-IFN- γ MAb, spleen counts were not different from controls until day 4 but then increased rapidly (Fig. 1). All mice treated with anti-IFN- γ MAb died on day 7 or 8. Similar results were obtained when anti-IFN- γ MAb was administered on day 1 instead of day 0 (data not shown).

The dose response to anti-IFN- γ MAb, measured in terms of spleen counts on day 7 of infection, showed that 25 μ g of MAb was sufficient to induce a significant increase in the number of bacteria in the spleen (Fig. 2).

Effects of anti-CD4 and anti-CD8 MAb on the early phase of *S. typhimurium* infection. IFN- γ can be secreted by a subpopulation of CD4 $^+$ T cells and by CD8 $^+$ T cells (24). Depletion of CD4 $^+$ or CD8 $^+$ T cells in vivo did not significantly modify spleen counts on day 7 (Table 1). In mice depleted of both CD4 $^+$ and CD8 $^+$ T cells, spleen counts were significantly higher than in controls ($P < 0.01$) (Table 1)

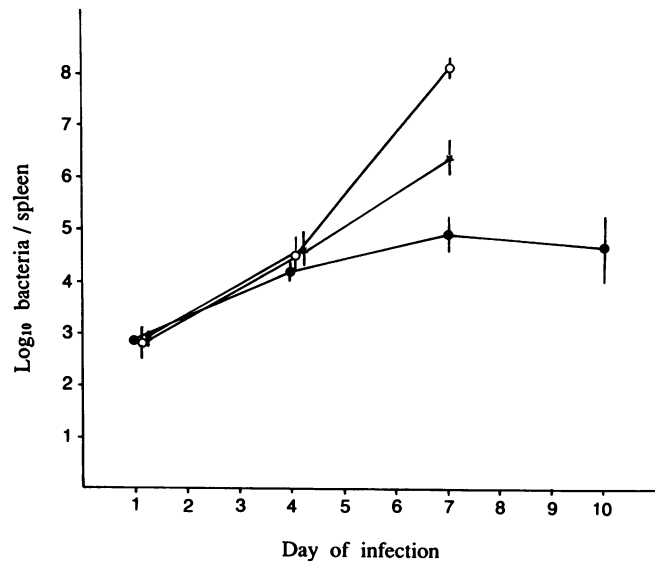


FIG. 1. Time course of infection in mice inoculated with 10^3 CFU of *S. typhimurium* C5 and treated at day 0 with anti-IFN- γ (○), anti-TNF- α (★), or control antibodies (●). Data are the geometric mean number \pm standard deviation of bacteria recovered from the spleens of three mice per group. On day 7, the difference between controls and mice treated with anti-IFN- γ or anti-TNF- α was statistically significant ($P < 0.01$).

but significantly lower ($P < 0.01$) than in mice treated with anti-IFN- γ antibodies in the same experiment (Table 2). These results suggest that during the first week of infection, IFN- γ is produced, at least in part, by cells other than T lymphocytes.

Effects of anti-TNF antibodies on *S. typhimurium* infection. Administration of 0.2 ml of anti-TNF antibodies 2 h after an i.v. injection of a sublethal dose of *S. typhimurium* had an effect similar to the administration of anti-IFN- γ antibodies; spleen counts were significantly increased on day 7 (Fig. 1), and the mice died a few days later. When anti-TNF- α (0.2 ml) and anti-IFN- γ (400 μ g) antibodies were administered

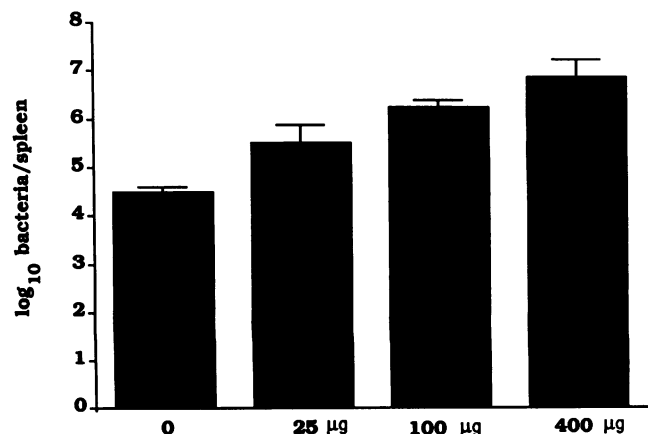


FIG. 2. Dose response to anti-IFN- γ MAb of mice infected with 10^3 CFU of *S. typhimurium* C5. The indicated doses of MAb were injected on day 0, and spleen counts were carried out on day 7. The difference between controls and mice treated with 25- μ g ($P < 0.05$) or higher doses ($P < 0.001$) of MAb was statistically significant.

TABLE 1. Effects of T-cell subset depletion in vivo on the early phase of *S. typhimurium* infection

Expt	Treatment ^a	Log ₁₀ bacteria/spleen	% CD4 ⁺	% CD8 ⁺
1	Control	4.44 ± 0.27	21.2 ± 1.8	13.7 ± 2.8
	Anti-CD4	4.75 ± 0.26	3.1 ± 1.8	15.2 ± 4.6
	Anti-CD8	4.96 ± 0.26	34.1 ± 4.8	1.5 ± 0.2
2	Control	4.18 ± 0.23	19.2 ± 2.7	11.8 ± 2.5
	Anti-CD4 + anti-CD8	4.95 ± 0.17 ^b	4.3 ± 1.7	2.3 ± 1.3

^a CBA mice were infected with 10⁵ CFU of *S. typhimurium* C5 and treated 1 h later with the indicated MAb. On day 7 (experiment 1) or day 6 (experiment 2), the number of viable bacteria and the percentage of CD4⁺ and CD8⁺ T cells per spleen were determined. Data are the mean ± standard deviation of results from three to four mice per group.

^b Statistically different from control ($P < 0.01$).

simultaneously, a synergistic interaction was observed (Table 2).

Effects of anti-IFN- γ and anti-TNF- α antibodies on resistance to reinfection with *S. typhimurium*. Mice immunized with an avirulent strain of *S. typhimurium* exhibit increased resistance to a secondary challenge with a virulent strain. CBA mice infected with 10⁶ CFU of the avirulent C5TS strain received 400 μ g of anti-IFN- γ MAb, 0.2 ml of anti-TNF- α antibodies, or both on day 6 postinfection. Controls received irrelevant antibodies. The following day, mice were challenged i.v. with 10⁵ CFU of *S. typhimurium* C5, and the number of viable bacteria per spleen was determined 3 days later. A representative result of four experiments is shown in Fig. 3. When given separately, anti-IFN- γ and anti-TNF- α antibodies induced a slight but significant increase in spleen counts, while the simultaneous administration of the two antibodies had a more marked effect. Bacterial counts in the liver followed a similar pattern (data not shown). The treatment did not, however, completely abrogate the protective effect of the avirulent strain.

Effects of anti-IFN- γ and anti-TNF- α antibodies on nonspecific resistance to reinfection. Previous studies have shown that nonspecific resistance to *L. monocytogenes* occurs during the first few days of *S. typhimurium* infection and persists as long as the animals carry the primary inoculum (31). CBA mice were infected with an avirulent strain of *S. typhimurium* C5TS and challenged by day 7 with a virulent strain of *L. monocytogenes*. One day before challenge,

TABLE 2. Interaction between anti-IFN- γ and anti-TNF- α antibodies in the depression of resistance to primary infection with *S. typhimurium*

Treatment ^a	Log ₁₀ bacteria/spleen ^b	P ^c
Control	4.18 ± 0.23	
Anti-IFN- γ	5.80 ± 0.22	<0.001
Anti-TNF- α	5.49 ± 0.57	<0.01
Anti-IFN- γ + anti-TNF- α	6.89 ± 0.10 ^d	<0.001

^a CBA mice were infected with 10⁵ CFU of *S. typhimurium* C5 and treated 1 h later with anti-IFN- γ (400 μ g) and anti-TNF (0.2 ml) separately and in association.

^b Results are the geometric mean number ± standard deviation of bacteria recovered on day 6 from the spleens of four mice per group.

^c P value versus control.

^d Bacterial counts were significantly higher in mice receiving both antibodies simultaneously than in those receiving either anti-IFN- γ ($P < 0.001$) or anti-TNF- α ($P < 0.01$) alone.

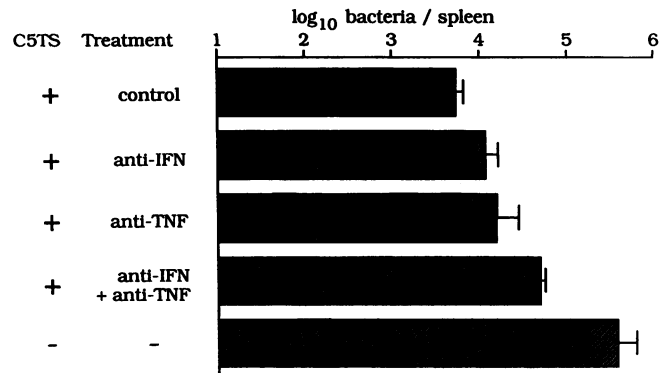


FIG. 3. Effects of anti-IFN- γ and anti-TNF- α antibodies on resistance to reinfection with *S. typhimurium*. CBA mice were infected with 10⁶ CFU of the avirulent *S. typhimurium* C5TS strain and treated on day 6 with the indicated antibodies. On day 7, they were challenged i.v. with 10⁵ CFU of *S. typhimurium* C5. Three days later, the number of viable C5 per spleen was determined. The increase in spleen counts above control values was statistically significant for mice treated with anti-IFN- γ ($P < 0.05$) and anti-TNF- α ($P < 0.05$) antibodies separately and in association ($P < 0.001$).

groups of mice were treated, as described above, with anti-IFN- γ and/or anti-TNF- α antibodies. The number of viable *L. monocytogenes* per spleen was determined 3 days after challenge. Figure 4 shows that the increased resistance to *L. monocytogenes* induced by *S. typhimurium* infection was significantly reduced by both anti-IFN- γ and anti-TNF- α antibodies and totally abrogated by their simultaneous administration.

DISCUSSION

Our results show that IFN- γ plays a critical role in the control of *S. typhimurium* infection. When anti-IFN- γ MAb was administered at the onset of a primary infection, spleen counts were significantly different between treated and con-

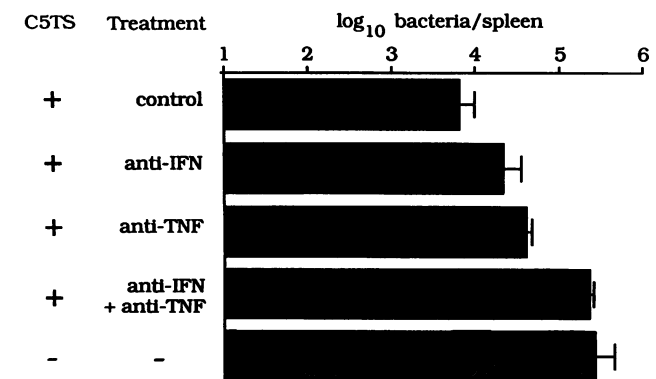


FIG. 4. Effects of anti-IFN- γ and anti-TNF- α antibodies on nonspecific resistance to reinfection. CBA mice were infected with 10⁶ CFU of *S. typhimurium* C5TS and treated on day 6 with the indicated antibodies. On day 7, they were challenged i.v. with 10⁵ CFU of *L. monocytogenes*. Three days later, the number of viable *L. monocytogenes* per spleen was determined. The increase in spleen counts above control values was statistically significant for mice treated with anti-IFN- γ ($P < 0.01$) and anti-TNF- α ($P < 0.001$) antibodies separately and in association ($P < 0.001$).

trol mice after 4 days, suggesting that IFN- γ production begins after a lag period of a few days. Bacterial growth plateaued over the following days in control animals, whereas it continued in mice treated with anti-IFN- γ , which died by day 7 or 8. Endogenous IFN- γ production was therefore necessary for survival.

Other studies have shown that exogenous IFN- γ increases resistance to *S. typhimurium* (23) and stimulates bactericidal activity of macrophages against this bacterium (19), although Van Dissel et al. (35) have published conflicting results. It has also been reported that anti-IFN- γ antibodies decrease resistance to primary infection with *S. typhimurium* (25) and *L. monocytogenes* (6).

It is not known which cell(s) produces IFN- γ in the early phase of *S. typhimurium* infection. Depletion of CD4⁺ or CD8⁺ T cells in vivo did not modify resistance to *S. typhimurium* infection in the early phase, confirming our previous studies (29). Depletion of both subpopulations slightly depressed resistance, but to a lesser extent than anti-IFN- γ treatment. These results suggest that the major source of IFN- γ is not T cells at this stage of infection. It is known that IFN- γ can be produced in mice lacking T cells (2, 36) and that natural killer cells can also be a source of IFN- γ (38). Bancroft et al. have shown that *scid* mice infected with *L. monocytogenes* produce IFN- γ by a T-cell-independent pathway involving natural killer cells (3). Further studies are needed to characterize the cells producing IFN- γ in *S. typhimurium* infection.

Exogenous TNF- α can increase resistance to *S. typhimurium* infection (27). Our results show that endogenous TNF- α production plays a role in the host response to *S. typhimurium* infection, since the administration of anti-TNF- α antibodies decreased resistance to infection. Other studies have shown that anti-TNF- α antibodies also decrease resistance to *L. monocytogenes* (13), *Chlamydia trachomatis* (37), and *Mycobacterium bovis* BCG (21) infections. TNF- α can activate the bactericidal activity of macrophages (5, 9) and seems to play a role in the development of granulomas (21).

Simultaneous treatment of infected mice with anti-IFN- γ and anti-TNF- α antibodies showed that IFN- γ and TNF- α acted synergistically to increase resistance to both *S. typhimurium* infection and *S. typhimurium* or *L. monocytogenes* reinfection. A synergistic interaction between IFN- γ and TNF- α has been reported in several other studies (14, 22, 26). IFN- γ increases the number of TNF- α receptors (1, 34) and the transcription of TNF- α mRNA (7) in macrophages. Recent studies have also shown that TNF- α is necessary for the production of IFN- γ by natural killer cells (3).

Taken together, our results show that IFN- γ and TNF- α are produced in the early phase of *S. typhimurium* infection and that this is of critical importance for the survival of the host. The capacity of IFN- γ and TNF- α to activate the bactericidal activity of macrophages is well established (5, 9, 28). It has been reported that macrophages can transfer resistance to *S. typhimurium* infection (20), and Hormaeche et al. have found that the plateau phase which occurs at the end of the first week of *S. typhimurium* infection is mediated by radiation-sensitive bone marrow-derived cells, but not T cells (16). Our results suggest that these cells need to be activated by IFN- γ and TNF- α . The production of cytokines seems to be an early mechanism of resistance which probably occurs before the specific immune response.

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