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Role of Gamma Interferon in Late Stages of Murine Salmonellosis

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Gamma interferon (IFN-γ) is an important mediator of natural resistance of mice to *Salmonella* species during the first week of infection, when it restricts the rate of intracellular growth of the bacteria but does not lead to their killing (A. Muotiala and P. H. Mäkelä, Microb. Pathog. 8:135–141, 1990). We used the experimental mouse salmonellosis model to investigate the role of IFN-γ in the later stages of a sublethal infection and the ensuing specific immunity. When anti-IFN-γ was administered starting 6 days after challenge, it did not prevent the cessation of intracellular bacterial growth and the formation of the plateau stage in the second week of infection. In addition, anti-IFN-γ given 14 and 16 days after challenge did not alter the elimination of the bacteria in the clearance stage in the third week of infection. When mice immunized 2 months previously with live vaccine were infected with virulent salmonellae, depletion of IFN-γ enhanced the early growth of the bacteria in the same manner as that seen in naive mice. However, when the immunized mice were infected with attenuated *aroA* bacteria, their clearance started immediately and was unaffected by IFN-γ depletion, demonstrating that IFN-γ is not required for the clearance. We conclude that IFN-γ restricts the rate of intracellular bacterial multiplication in the first week of *Salmonella* infection in both naive and immune mice but is not a mediator of bacterial clearance in either naive or immunized mice.

Mouse typhoid is an extensively used experimental model for human typhoid fever. When mice are challenged with a sublethal dose of *Salmonella enterica* serovar Typhimurium or Enteritidis, the infection develops over a period of weeks in several clearly identifiable stages. During the natural infection, an initial stage of invasion of the bacteria through the intestinal wall is followed by their localization in cells, presumably macrophages, first in local lymph nodes and soon predominantly in the liver and spleen (3, 7, 14). When the experimental infection is initiated by intravenous (i.v.) injection (Fig. 1), these events are largely bypassed and the bacteria localize within 4 to 6 h in the liver and spleen. In the second stage, bacterial growth and multiplication take place over several days intracellularly in the liver and spleen. Third, this multiplication is suppressed so that the numbers of bacteria in these organs remain unchanged. This plateau stage lasts for approximately a week, coinciding with a profound suppression of responsiveness of lymphoid cells to mitogenic stimuli (13). It is followed by a slow clearance of the bacteria, presumably mediated by the developing immunity. The final stage is a long-lasting state of specific immunity. If a nonreplicating strain, for example a nutritionally attenuated *aroA* mutant, is used for challenge (Fig. 1), there is no or very little multiplication of the bacteria, but the plateau is followed by clearance and immunity with the same kinetics described above (20).

Gamma interferon (IFN-γ) plays an important role at the early, bacterial growth stage by restricting the rate of multiplication of the bacteria (Fig. 1 [inset]) (21, 23, 26). The action of IFN-γ seems to be bacteriostatic, not bactericidal, since the infection kinetics of the nonreplicating *aroA* strain were not altered (21). However, all of these studies have focused on the growth stage of the infection. The host defense mechanisms responsible for the formation of the plateau or the ensuing bacterial clearance have not been investigated, the first open question being whether IFN-γ also stimulates killing and elimination of the intracellular salmonellae at these stages.

During the first weeks of *Salmonella* infection, mice are resistant to reinfection (12). This is a nonspecific resistance affecting, e.g., *Listeria monocytogenes*, and has recently been shown to be mediated by natural killer (NK) cells (12, 31). It should not be confused with the specific immunity developing more slowly but lasting for at least a year (12, 14, 20).

The mechanisms of the specific immunity are poorly understood. In the initial stage of infection, the bacteria are accessible to antibodies, and anti-O but not antibodies to rough bacteria are protective, although to a limited extent (20, 29, 34). Later, antibodies do not have any effect on the intracellular bacteria (15, 19). $CD4^+$ T cells have been shown to be important for protective immunity following infection by a live attenuated vaccine strain or by a sublethal dose of virulent salmonellae (10, 22). IFN-γ produced by the $CD4^+$ T cells would be a logical mediator of protective immunity through activation of macrophages for killing of the intracellular bacteria (15), but direct evidence of this is lacking. IFN-γ has been shown to activate macrophages for such anti-*Salmonella* activity in vitro, but the relevance of the in vitro studies to events during infection has been questioned (11, 27).

The present series of experiments was therefore planned to define the role of IFN-γ as a mediator at the later stages of sublethal serovar Typhimurium infection, including the plateau and clearance stages as well as subsequent specific immunity. The experimental design was based on neutralizing the effect of IFN-γ in vivo by monoclonal antibodies and on following the course of the infection by enumerating the live serovar Typhimurium bacteria in the liver and spleen at different intervals after i.v. challenge by either a mouse-virulent strain (SL2965) or a nonreplicating *aroA* mutant

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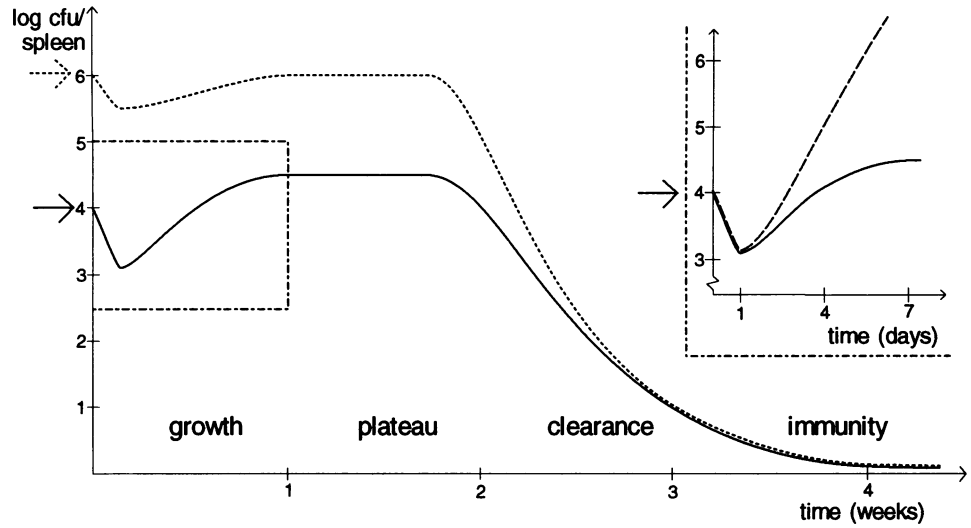

FIG. 1. The kinetics of a sublethal serovar Typhimurium infection in mice. ——, virulent strain, . . ., nonreplicative *aroA* strain (20); – – – (in inset), anti-IFN-γ-treated mice (21). Arrows show the challenge dose, which was injected i.v.

(SL3261) of serovar Typhimurium (8, 21). The data show, somewhat surprisingly, that IFN-γ was not the mediator of bacterial elimination that occurred at the later stages of infection or during the subsequent immunity.

MATERIALS AND METHODS

Mice. (CBA × C57BL/6)F_1 hybrid female mice, bred at the National Public Health Institute, Helsinki, Finland, were used at the age of 8 to 10 weeks.

Bacteria and growth conditions. The *S. enterica* serovar Typhimurium strains used were SL2965 (33), SL3261 (8), and *his*-515 (24). Strain SL2965 is a virulent strain with a 50% lethal dose of 10^5 by the i.v. route in the mice used (20). Strain SL3261 is an *aroA* mutant strain, which is unable to replicate in mouse tissues because of its inability to synthesize several nutritionally important metabolites of the aromatic amino acid pathway (32). Strain *his*-515 is a rough mutant, with the Ra chemotype, because of a deletion in the *rfb* gene cluster (24). As a vaccine, it induces protective immunity in mice (20). All strains were mouse passaged and stored in 1% skim milk (5) at −70°C until used. The bacteria were grown at 37°C in L broth (17) under aeration into late exponential phase and diluted in saline to the appropriate concentration. Viable organisms were counted by plating serial dilutions on L agar plates.

Infection. The i.v. challenge was given in a volume of 200 μl. The outcome of the infection was evaluated by counting the viable bacteria in the liver and spleen as CFU at the indicated days after challenge as described previously (28). Each point represents the geometric mean of the results from three mice, with the standard error of the mean (SE) indicated. The statistical significance of differences between groups of mice was calculated by Student's *t* test on log-transformed data.

Anti-IFN-γ treatment. Hamster anti-mouse IFN-γ monoclonal antibody (25 μg) (Genzyme Corporation, Boston, Mass.) diluted in 200 μl of 0.1% bovine serum albumin in phosphate-buffered saline was injected intraperitoneally (i.p.) as indicated. Age-matched control mice received the diluent only.

Assay for IFN-γ production. Mononuclear cells were purified from the teased spleens (pools of three) by Ficoll-Paque centrifugation (Pharmacia LKB, Uppsala, Sweden) and resuspended in standard RPMI 1640 medium containing 10% fetal bovine serum, L-glutamine (1 mM), penicillin G (50 U/ml), streptomycin (50 μg/ml), and kanamycin (50 μg/ml). The cells were plated in 96-well round-bottom microtiter plates in 200 μl at 2×10^5 cells per well in the absence or presence of different concentrations of *Salmonella* envelopes from the rough *his*-515 strain prepared as described by Nurminen (25) and stored at −20°C at a protein concentration of 5 mg/ml in 1% sodium dodecyl sulfate. Triplicate cultures were incubated at 37°C for 48 h in a CO_2-enriched atmosphere (5% CO_2–95% air). Supernatants from the wells were pooled and frozen at −20°C until IFN-γ activity was assayed by the InterTest-γ mouse IFN-γ enzyme-linked immunosorbent assay kit according to the instructions of the supplier (Genzyme Corporation).

RESULTS AND DISCUSSION

The plateau stage of infection. Mice were challenged i.v. with the mouse-virulent serovar Typhimurium strain SL2965 at a sublethal dose of $10^{4.4}$ bacteria (0.25 times the 50% lethal dose). After initial undisturbed progress of the infection, the experimental group was injected with anti-IFN-γ on days 6, 8, and 10, while the control group received the diluent only (Fig. 2). The control group was expected to reach the plateau stage by day 7, and no change in bacterial numbers between days 7 and 13 was also observed. By contrast, the numbers of bacteria in the tissues of the anti-IFN-γ-treated mice continued to increase until day 10. By this time, the anti-IFN-γ-treated mice also reached the plateau stage and the numbers of bacteria no longer increased. Consequently, the bacterial numbers in the organs at the time of the plateau were approximately 10-fold higher in the anti-IFN-γ-treated mice than in the controls.

The fact that anti-IFN-γ delayed but did not prevent the formation of the plateau shows that IFN-γ is not the decisive factor that restricts bacterial growth during the plateau stage, which therefore must be due to another defense mechanism(s). This is in agreement with findings of Hormaeche et al. and Maskell et al. (9, 15), who showed that the

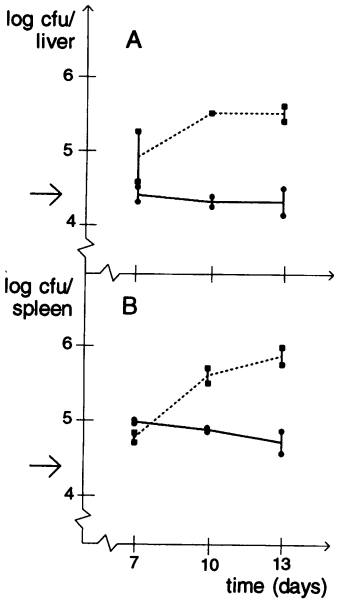

FIG. 2. Effect of anti-IFN-γ treatment at the plateau stage of mouse salmonellosis. Arrows show the challenge dose of SL2965 given i.v. Mice were injected i.p. with 25 μg of anti-IFN-γ (– – –) or diluent (——) on days 6, 8, and 10 after challenge. They were sacrificed 7, 10, and 13 days after challenge, and the numbers of CFU in the livers (A) and spleens (B) were determined. Each point represents the geometric mean for three mice; bars indicate the SE. The level of the plateau was significantly higher in anti-IFN-γ-treated mice ($P < 0.001$).

formation of the plateau but not the preceding growth phase is dependent on radiation-sensitive cells that are not T cells. The effect of anti-IFN-γ between days 7 and 10 of the experiment in Fig. 2 may indicate that at this time two separate host defense mechanisms regulated bacterial growth: IFN-γ restricting the rate of growth, and a separate, plateau-specific mechanism that eventually stopped the growth.

The clearance stage of infection. To investigate the clearance stage, the mice were given the same sublethal challenge dose of the virulent bacteria and left undisturbed for 14 days, i.e., until the expected time of clearance of the infection according to Fig. 1. The experimental group of mice was treated with anti-IFN-γ on days 14 and 16 and compared with the control group treated with diluent. The numbers of viable bacteria in the spleen (Fig. 3) and liver (data not shown) declined from day 15 to day 19 in both groups in virtually the same manner, demonstrating that IFN-γ does not play a role in the clearance stage of the infection. Thus, other antibacterial mechanisms must be responsible for the elimination of the bacteria. Previous studies have shown that T cells are required for this clearance (22); the present data say that their effect is not mediated via IFN-γ.

The stage of specific immunity. To study the role of IFN-γ in specific cell-mediated immunity that remains for months after sublethal infection, we immunized the mice with an attenuated rough strain *his*-515, which has a chromosomal deletion extending to the *rfb* genes and thus preventing the synthesis of any O antigen (24). The *his*-515 bacteria are eliminated with similar kinetics as the *aroA* strain shown in Fig. 1; the protective immunity lasts for at least 1 year and is dependent on cellular mechanisms, since no anti-O antibodies are produced and passive administration of anti-*his*-515 does not have an effect on the kinetics of the *Salmonella* infection (20). The mice were allowed to rest for at least 2 months to ensure total clearance of the immunizing strain (20) before challenge.

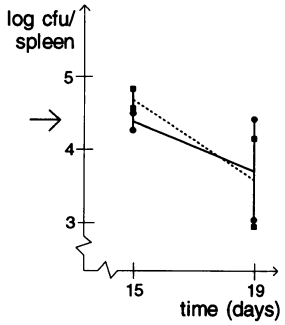

FIG. 3. Effect of anti-IFN-γ on the clearance stage of mouse salmonellosis. Arrow shows the challenge dose of SL2965 given i.v. Mice were injected i.p. with 25 μg of anti-IFN-γ (– – –) or diluent (——) 14 and 16 days after challenge. Mice were sacrificed 15 and 19 days after challenge, and the numbers of CFU in the spleens were determined. Each point represents the geometric mean for three mice; bars indicate the SE.

The kinetics after challenge of the immunized mice with the virulent SL2965 strain at a dose of 1.3 times the 50% lethal dose (Fig. 4A, dots) were compared with the kinetics in naive, nonimmune mice (Fig. 4A, squares). In the naive mice, the bacteria replicated, as expected with this challenge dose, up to the point that by day 7 already two of the mice had died. In the immunized mice, the numbers of bacteria in the spleens 1 day after challenge were approximately 20% of those in naive mice, i.e., the initial drop in bacterial numbers relative to the challenge dose was increased. The difference between naive and vaccinated mice increased on subsequent days, so that on day 4 after challenge the number of viable bacteria in the spleens of vaccinated mice was 1% and on day 7 it was less than 0.01% of that in naive mice. Thus, protective immunity could be detected already on day 1 and resulted in a net decrease in bacterial numbers after day 4. In other words, the clearance stage in the immunized mice started very early in the infection and rapidly counterbalanced the bacterial growth that had started between days 1 and 4. The initial drop in bacterial numbers that was magnified in the immunized animals was not affected by anti-IFN-γ given just before challenge. The same lack of effect of anti-IFN-γ has been shown to occur in unimmunized mice, consistent with a slow start of IFN-γ synthesis 1 to 2 days after challenge (21, 23, 26).

The kinetics after challenge with the nonreplicative SL3261 *aroA* bacteria are shown in Fig. 4B. In the naive mice, the bacterial numbers remained almost constant throughout the follow-up period (Fig. 4B, squares), corresponding to the data shown in Fig. 1. In the immunized mice (Fig. 4B, dots), there was, as with the virulent challenge, an increased initial drop in bacterial numbers to approximately 10% of those seen in naive mice on day 1. In contrast to the virulent strain, there was no bacterial growth in the next few days but instead a continuous, slow decrease in the numbers of the bacteria in the immunized mice, suggesting that immune clearance had already started by day 1, i.e., even earlier than indicated after challenge with the virulent strain.

When the immunized *aroA*-challenged mice were treated

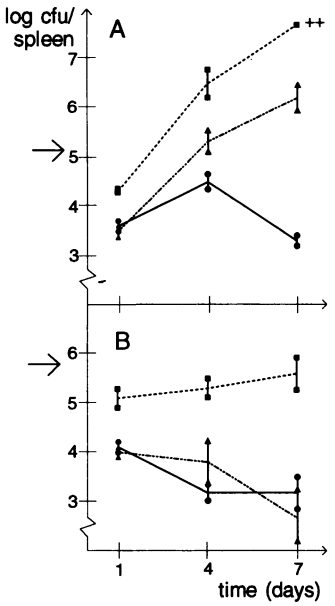

FIG. 4. Effect of anti-IFN-γ during the stage of specific immunity. Arrows show the challenge dose of virulent SL2965 (A) or nonreplicative *aroA* SL3261 (B) given i.v. on day 0. Squares, data from control animals (naive mice injected i.p. with diluent but no antibodies); triangles, data from mice immunized at least 2 months previously with the attenuated rough *his*-515 strain and injected i.p. with 25 μg of anti-IFN-γ 1 h before and 2 days after challenge; dots, data on similarly immunized mice injected with diluent instead of antibodies. Mice were sacrificed 1, 4, and 7 days after challenge, and the numbers of CFU in the spleens were determined. Each point represents the geometric mean for three mice, and bars indicate the SE. In one group, two of the three mice had already died by day 7 (++), and, since deaths are known to occur after the number of bacteria has reached 10^8 (14), this was used as a point estimate for the dead mice. Statistically significant ($P < 0.01$) differences between naive mice and either of the immunized groups were seen at each time point with both challenge organisms (A and B). On days 4 and 7, there was also a statistically significant ($P < 0.01$) difference between the two immunized groups treated either with anti-IFN-γ or diluent shown in panel A (virulent challenge).

with anti-IFN-γ given before and 2 days after challenge, the numbers of bacteria in the organs decreased just as they did without this treatment (Fig. 4B, triangles). This finding was precisely the same as that seen during the clearance stage in naive mice (Fig. 3), consistent with the hypothesis that elimination of the bacteria is mediated by the same mechanisms of cell-mediated immunity both in the clearance stage and in the stage of specific immunity. In both cases, the T-cell-dependent effects (22) are not mediated by IFN-γ (present data).

By contrast, treatment with anti-IFN-γ of the immunized mice challenged with the virulent strain had a profound effect on the course of the infection (Fig. 4A, triangles). After the initial drop, the apparent rate of bacterial growth was faster than in the immunized, diluent-treated mice (Fig. 4A, dots) between days 1 and 4 and continued to the end of the experiment (day 7), at which time the immunized, IFN-γ-replete mice had already started elimination of the bacteria. The infection in the immunized, IFN-γ-depleted mice still remained restricted in comparison with naive mice without (Fig. 4A, squares) or with (Fig. 1 [inset]) anti-IFN-γ. We suggest that in the immunized mice, two competing and

TABLE 1. Production of IFN-γ by spleen mononuclear cells stimulated with serovar Typhimurium envelope[a]

Mouse type	Log CFU/spleen	IFN-γ production (pg/ml) with the following amts of envelope (μg/well):		
		0	0.1	1.0
Uninfected naive	0	0	0	600
Infected naive	5.5	0	4,800	7,700
Infected immune	3.6	0	2,800	4,800

[a] Spleens were removed 2 days after i.v. challenge with 10^5 bacteria (SL2965). Mononuclear cells (2×10^5 cells per 200-μl well) were cultured for 48 h in the presence of the indicated amounts of envelope. Supernatants were used for IFN-γ measurement.

opposite processes occurred between days 1 and 7 after challenge: (i) bacterial growth, restricted by IFN-γ, in a manner qualitatively and quantitatively similar to that seen in naive animals and (ii) bacterial killing and elimination mediated by immune mechanisms. After challenge of the immunized mice with the nonreplicating *aroA* strain, only the second process, bacterial killing and elimination, would be relevant, and indeed this challenge provides an experimental setup to study this process undisturbed by bacterial growth. This experiment (Fig. 4B) clearly showed that IFN-γ does not participate in the process of bacterial elimination. However, the data in Fig. 4A suggest that IFN-γ may have to act early in the infection in order to allow the subsequent events—formation of plateau and clearance—to occur.

Since the mechanisms restricting *Salmonella* growth in the first days of infection in naive or immune mice are not T-cell dependent (9, 12, 22) but mediated by NK cells (31), these are in fact likely to be the major producers of IFN-γ at this stage. However, $CD4^+$ TH1 cells are also known to be important producers of IFN-γ (18), and they would be expected to be stimulated soon after challenge in the immunized mice. Therefore, we found it of interest to ask whether the immunized mice would produce more IFN-γ than naive mice after challenge. To do this, we chose the sensitive method of measuring IFN-γ production in vitro by spleen cells removed from the mice 2 days after challenge (6). The cells were tested as such and after stimulation with two different doses of a *Salmonella* envelope preparation (Table 1). Cells of both immunized and naive infected mice produced considerable amounts of IFN-γ when stimulated with either dose of the envelope but not without in vitro stimulation. The amount of IFN-γ produced was of the same order of magnitude in both groups of mice; the somewhat lower amount of IFN-γ produced by cells of the immunized mice most likely reflects the lower number of bacteria in the organs of these mice ($10^{3.6}$ versus $10^{5.5}$ CFU per spleen).

Conclusions. We showed here that anti-IFN-γ treatment starting 6 days after challenge of mice with a sublethal dose of virulent serovar Typhimurium cells did not prevent the establishment of the plateau stage (second week) or subsequent clearance (third week) of the bacteria. Neither did it have an effect on the clearance of the nonreplicating *aroA* bacteria, which started in the naive mice in the third week of infection and soon after challenge in the immunized mice. In contrast, IFN-γ depletion did increase the rate of bacterial growth in the first week of infection with virulent salmonellae in both naive and immunized mice.

In vivo neutralization of IFN-γ or other cytokines by monoclonal antibodies has proven an efficient means of

testing the effect of these cytokines on infection (2, 4, 23). It is better than injection of the cytokines because of their often short half-lives and local effect. On the other hand, it can always be argued that the anti-IFN-γ injected did not manage to neutralize all IFN-γ present. This does not seem likely in the present study, since the dose of the monoclonal antibody used was two or three times the dose that was previously found sufficient to neutralize the effect of IFN-γ in experiments with 10-fold-higher bacterial loads (19, 21). The same monoclonal antibody has also been shown to be able to block the resolution of intracellular *L. monocytogenes* infection in mice (4).

Therefore, we conclude that IFN-γ is the main mediator of restriction of bacterial growth in the early stages (first week) of *Salmonella* infection in both naive and immunized mice. Tumor necrosis factor alpha also restricts the growth of salmonellae but seems to act at a somewhat later stage (16, 23, 35). The cell type producing IFN-γ during the first week of salmonellosis has not been identified, but our data are consistent with the possibility that it would be the NK cell. These cells have been shown to be an important source of IFN-γ in *L. monocytogenes* infection (1), and NK or NK-like cells have been shown to be involved in early resistance to salmonellosis in both naive and immunized mice (12, 31).

We further conclude that IFN-γ is not a direct mediator of bactericidal functions even in immunized mice. We would also like to point out that there was no indication of increased production of IFN-γ or increased sensitivity to it in immune mice in which bacterial clearance had already started, compared with naive mice in which clearance would start only 2 weeks later. It is, therefore, obvious that mechanisms other than IFN-γ must operate in and be responsible for the plateau, clearance, and immunity stages of salmonellosis, as well as for the initial drop in the first hours of infection. Various pieces of evidence have already pointed at different mechanisms being responsible for the different stages (9, 15, 16, 22, 23). In the initial stage, resident macrophages seem to be involved (3, 30), whereas in the latest stages T-cell-dependent immune mechanisms (10, 22) certainly have a role. The elucidation of these latter mechanisms may shed new light on the mechanism of action of live *Salmonella* vaccines and, by comparison, also on effector mechanisms of immunity in other intracellular infections, including malaria and leishmaniasis.

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