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Systemic Infection of Mice by Wild-Type but Not Spv⁻ *Salmonella typhimurium* Is Enhanced by Neutralization of Gamma Interferon and Tumor Necrosis Factor Alpha

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The *spv* genes of the virulence plasmid of *Salmonella typhimurium* and other nontyphoidal serovars of *S. enterica* are involved in systemic infection by increasing the replication rate of the bacteria in host tissues beyond the intestines. We considered the possibility that the Spv virulence function is to evade suppression by the host response to infection. To examine this possibility, gamma interferon (IFN-γ) and/or tumor necrosis factor alpha (TNF-α) were neutralized in BALB/c mice by intraperitoneal administration of monoclonal antibodies. Neutralization of IFN-γ and/or TNF-α resulted in increased splenic infection with wild-type salmonellae after oral inoculation; however, Spv⁻ salmonellae were defective at increasing splenic infection in cytokine-depleted mice. The use of a temperature-sensitive marker plasmid, pHSG422, indicated that neutralization of IFN-γ caused less killing of wild-type *S. typhimurium*, while neutralization of TNF-α resulted in an increased in vivo replication rate for wild-type salmonellae. These results demonstrate that the Spv virulence function is not to evade suppression of bacterial infection normally mediated by IFN-γ or TNF-α.

The *spv* genes are encoded on the virulence plasmids of serovars of *Salmonella enterica* other than *S. typhi*, which have the potential to cause systemic disease in animals (18). The *S. typhimurium spv* genes enable the bacteria to infect the spleen and liver after oral inoculation (17) by increasing the replication rate of the bacteria within host cells of mice (19). The DNA sequences encoding the *spv* genes of several serovars have been determined, but the deduced sequences of the Spv proteins have not aided in the elucidation of their functions, other than that of the positive regulatory protein SpvR (18). Furthermore, the regulation of *spv* gene expression has been extensively studied (16, 18). The *spv* genes are induced during different stresses, including starvation and the stationary phase of growth (16, 18), and within host cells (14, 38, 42). However, the molecular and cellular virulence mechanisms encoded by the *spv* genes resulting in increased replication in vivo are unknown.

In their initial report on the plasmid-mediated virulence of *S. typhimurium*, Gulig and Curtiss showed that plasmid-containing and plasmid-cured *S. typhimurium* did not begin to diverge in splenic infection until 3 days after intravenous (i.v.) inoculation of mice (17). This result suggested that either the bacteria underwent some phenotypic change to enable the Spv function to become important or, conversely, the murine host changed its response to the infection to select against the replication of Spv⁻ salmonellae. The present study was therefore undertaken to examine if the production of cytokines which would be expected to be produced early in the disease process could be responsible for the suppression of replication of Spv⁻ salmonellae. The two cytokines for initial study were gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α).

IFN-γ and TNF-α are known to be of critical importance in the host response to infection by salmonellae, as well as other microbial pathogens of infected mice. Studies have revolved around three general types of models: neutralization of cytokines by administration of antibodies; use of genetically defective, transgenic knockout mice; and administration of recombinant cytokines. Focussing on the roles of IFN-γ and TNF-α in murine salmonellosis presents a complex and sometimes contradictory picture. This is because the genotype of the mouse host can vary between that of mice homozygous for the *Ity*^{r} allele (resistant) and that of mice homozygous for the *Ity*^{s} allele (sensitive). Mice have been inoculated either orally, subcutaneously, i.v., or intraperitoneally (i.p.). Some studies have focussed on effects on initial or primary infection, while others have examined effects on resistance to secondary challenge. However, it is clear that neutralization of IFN-γ or TNF-α has a profound effect on increasing salmonella infection at different times, depending on the mouse strain and the route of inoculation (22, 25–28, 30, 32, 33, 40). Furthermore, the measured effects on either replication of the bacteria or killing of bacteria by the host are not always in agreement.

Our studies examined the roles of IFN-γ and TNF-α in *Ity*^{s} BALB/c mice inoculated orally with Spv⁺ or Spv⁻ *S. typhimurium*. With oral inoculation versus parenteral inoculation, the bacteria must overcome all of the natural host defenses in a relevant time frame in relation to the production of host response components. We examined if IFN-γ and/or TNF-α were involved in the suppression of replication of Spv⁻ *S. typhimurium* in mice. If either cytokine caused host cells to differentially suppress the replication of Spv⁻ salmonellae, then neutralization of that cytokine would result in the Spv⁻ strain becoming more equal in infection to the wild-type strain in cytokine-depleted mice. Depletion of IFN-γ and/or TNF-α

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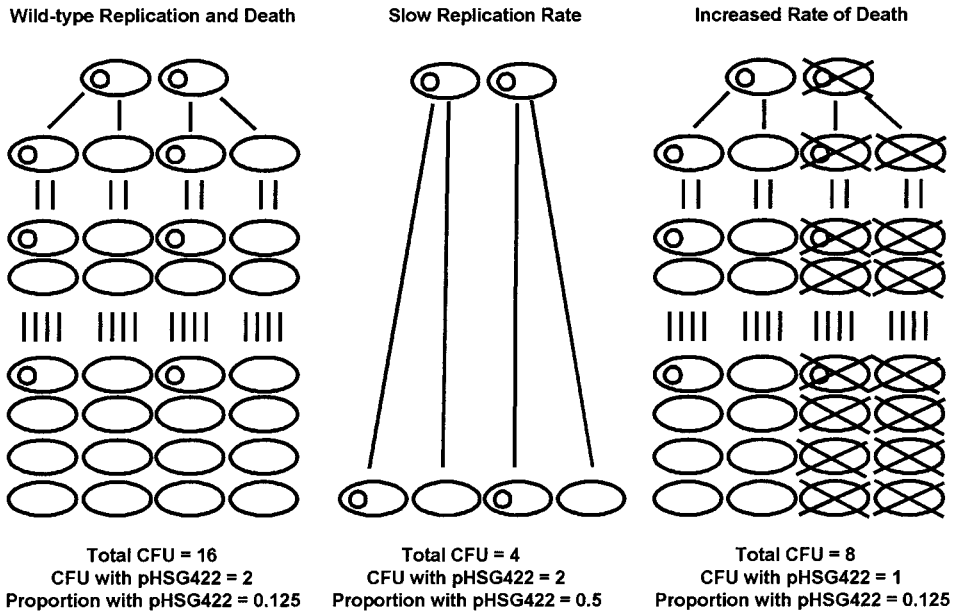

FIG. 1. Use of plasmid pHSG422 to differentiate the replication rate from the death rate of salmonellae in mice. Each of the three representative populations of salmonellae begins with two cells with 100% carriage of pHSG422 (small circle). In the example at the left for normal replication and killing, three divisions with no death yields 16 cells. Since pHSG422 did not replicate at body temperature, only 2 of the 16 cells (0.125 proportion) carry the original pHSG422 plasmids. In the center, with a slower replication rate in vivo, only one division occurs in the same time period. Four cells result, two of which carry the original pHSG422 plasmids and two of which do not (0.5 proportion). At the right, with a normal replication rate but increased killing, half of the cells are killed by the host (crossed-out cells), making the total cell count eight. The proportion of the population carrying pHSG422 is still 0.125; however, the number of cells carrying pHSG422 is halved to one. Therefore, the proportion of bacteria carrying pHSG422 is an indication of the growth rate, while the number of cells carrying pHSG422 is an indication of the death rate.

greatly increased systemic infection by wild-type *S. typhimurium*. However, depletion of either or both of these cytokines had a much reduced, usually statistically insignificant, effect on Spv$^-$ salmonellae. Therefore, production of IFN-γ or TNF-α is not essential for selection against Spv$^-$ *S. typhimurium*, or, stated another way, the Spv virulence function is not to evade host responses mediated by IFN-γ or TNF-α. Instead, Spv$^-$ salmonellae have such attenuated infection potential in vivo that they are mostly unable to take advantage of the lack of IFN-γ or TNF-α.

(A preliminary report of these results was presented at the 95th General Meeting of the American Society for Microbiology [29]).

MATERIALS AND METHODS

Bacterial strains and culture. The *S. typhimurium* strains used in this study were as follows. Wild-type, Spv$^+$ strains of the SR-11 line were χ3181, χ3306 (*gyrA1816*), and χ3456 (pStSR100::Tn*minitet*) (17). The plasmid-cured, Spv$^-$ strain used was χ3337 (pStSR100$^-$ *gyrA1816*) (17). A Δ*spv*::*tet* Spv$^-$ strain, UF110, was χ3181 with a 6.3-kb *Cla*I fragment of the *spv* region encompassing sequences upstream of *spvR* to within *spvD* replaced with a *tet* gene derived from pBR322 (15). To differentiate effects of treatments of mice on growth versus killing of salmonellae during infection, the marker plasmid pHSG422 (4, 20) was used as previously described (19) in strains χ3456 and UF110. The use of this plasmid and interpretation of data are graphically depicted in Fig. 1.

Unless noted otherwise, bacteria were grown in L broth (LB) (24) at 37°C to exponential phase with an optical density at 600 nm of approximately 0.5 as shaking cultures derived by a 1:20 dilution of a standing overnight culture in LB. For culture of strains containing pHSG422, bacteria were incubated at 30°C as previously described (19). The solid medium used was LB with 0.15% (wt/vol) L agar. Antibiotics were included as appropriate, at the following concentrations (micrograms per milliliter): nalidixic acid, 25; tetracycline, 12.5 (for all strains except UF110, which was selected by using 7 μg/ml); chloramphenicol, 30; ampicillin, 100; kanamycin, 33.

Infection of mice. Specific-pathogen-free BALB/c mice were purchased from Charles River (Wilmington, Mass.). Oral inoculation of mice with 10^8 CFU of salmonellae was performed exactly as previously described (19). At 4 or 5 days postinoculation, mice were euthanized by carbon dioxide asphyxiation and their spleens and livers were removed. A portion of tissue was collected for histology, and the remaining tissue was homogenized in buffered saline containing 0.01% (wt/vol) gelatin (8) and plated on L agar containing appropriate antibiotics. Infections are reported as $\log_{10}$ total splenic CFU or CFU per gram of liver.

Anticytokine antibodies and treatment of mice. Hamster monoclonal antibodies to murine IFN-γ (H22.1) (5) and TNF-α (TN3-19.12) (39) and an isotype-matched control monoclonal antibody (6C8) were initially provided by Robert Schreiber, Washington University, St. Louis, Mo., as purified immunoglobulin. For the data presented here, additional monoclonal antibodies were purified from hybridoma cell lines obtained from the American Type Culture Collection through the release by DNAX as follows. The anti-IFN-γ monoclonal antibody was XMG1.2 (6), the anti-TNF-α monoclonal antibody was MP6-XT22.11 (1), and the isotype-matched control monoclonal antibody was R187, which is directed against the murine leukemia virus p30 gag protein (7). Purified immunoglobulin was obtained from hybridoma culture supernatants by using Dulbecco modified Eagle medium or RPMI medium containing immunoglobulin-free fetal bovine serum (Life Technologies). Supernatants were passaged over a protein G-Sepharose affinity column (Sigma), eluted with 0.1 M glycine-HCl (pH 2.7), and neutralized with 1 M Tris-HCl (pH 10). The purity of the antibody preparations was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

For the data presented here, mice were injected i.p. with 200 μg of purified antibody at the time of food and water deprivation on the day of oral inoculation. In preliminary experiments, mice were injected i.p. with 100 μg of the antibodies from Robert Schreiber.

Histological analysis of infected tissues. For histological examination, a portion of infected tissues was fixed in 10% (vol/vol) buffered formalin and then embedded in paraffin. Tissue sections approximately 5 μm thick were prepared and mounted on glass slides by the University of Florida Department of Pathology, Immunology, and Laboratory Medicine Diagnostic Referral Laboratory. Slides were stained with hematoxylin plus eosin and scored for pathology and host cellular response to infection. Pathology was scored in a blinded manner.

Statistical analysis. Differences in mean $\log_{10}$ CFU ± standard deviation were examined by using a two-tailed Student *t* test.

RESULTS AND DISCUSSION

Comparison of splenic infection by wild-type and virulence plasmid-cured *S. typhimurium* in mice depleted of IFN-γ or TNF-α. If the function of the *spv* genes was to enable salmonellae to overcome suppression of growth regulated by the cytokines IFN-γ and TNF-α, then neutralization of these cy-

TABLE 1. Neutralization of IFN-γ and TNF-α increases splenic infection by wild-type *S. typhimurium*[a]

Antibody treatment	Mean $\log_{10}$ splenic CFU ± SD (*P* value)	
	Wild type	Plasmid cured
Control	4.6 ± 1.3	2.7 ± 1.2 (<0.01)[b]
Anti-IFN-γ	7.4 ± 0.5 (<0.005)[c]	3.6 ± 0.9 (NS)[c]
Anti-TNF-α	6.5 ± 1.1 (<0.01)[c]	3.0 ± 1.1 (NS)[c]

[a] Mice were injected i.p. with monoclonal antibodies on the day of oral inoculation with either wild-type *S. typhimurium* χ3456 or virulence plasmid-cured *S. typhimurium* χ3337. Five days later, splenic CFU were measured. Results are combined from two experiments. There were eight mice in all groups except the anti-IFN-γ–wild-type (there were six mice due to two deaths) and anti-TNF-α–wild-type (there were seven mice due to one death) groups.

[b] *P* value compared with wild-type strain.

[c] *P* value compared with corresponding control antibody-treated mice.

tokines in mice would relieve the differential suppression of virulence plasmid-cured *S. typhimurium*, thereby enabling the strain to act more like the Spv^+ parental strain. We therefore injected mice i.p. with a neutralizing monoclonal antibody to IFN-γ or TNF-α or an isotype-matched control monoclonal antibody. Mice were orally inoculated with either wild-type *S. typhimurium* χ3456 or plasmid-cured χ3337. Five days later, CFU of salmonellae in spleens were enumerated (Table 1). In control-treated mice, plasmid-containing χ3456 was recovered at 79-fold higher levels than plasmid-cured χ3337 ($P < 0.01$). This result is typical for the difference in splenic CFU between plasmid-containing and cured *S. typhimurium* after oral inoculation (17, 19). In mice that were treated with an anti-IFN-γ antibody, recovery of plasmid-containing χ3456 was increased 631-fold over that in control mice ($P < 0.005$). Mice in which TNF-α was neutralized experienced a 79-fold increase in splenic CFU of χ3456 over the control mice ($P < 0.01$). In contrast, neutralization of either IFN-γ or TNF-α resulted in eight- and twofold increases, respectively, in splenic infection by virulence plasmid-cured χ3337. These increases were not statistically significant.

These results confirm that both IFN-γ and TNF-α are essential for the inhibition of systemic infection by wild-type *S. typhimurium* in orally inoculated mice. Ramarathinam et al. (37) performed studies most related to ours by orally inoculating *S. typhimurium* into BALB/c mice treated with a monoclonal antibody to IFN-γ. They reported large increases in the numbers of both splenic and hepatic CFU of wild-type *S. typhimurium* 4 days postinoculation. Muotiala and Makela (31) and Nauciel and Espinasse-Maes (33) reported that anti-IFN-γ antibody treatment caused an increase in the number of splenic CFU of *S. typhimurium* inoculated i.v. into Ity^r mice 4 days postinoculation. Similar to our results obtained with Spv^- *S. typhimurium*, the number of splenic CFU of nonreplicating $AroA^-$ *S. typhimurium* was not increased by neutralization of IFN-γ (31). Administration of an anti-IFN-γ antibody more than 6 days postinoculation delayed the onset of, but did not eliminate, the plateau phase of salmonella infection in Ity^r mice (32). Their conclusion was that IFN-γ primarily caused bacteriostasis in mice. Tite et al. (40) reported that neutralization of TNF-α resulted in a 100-fold increase in the number of splenic CFU of *S. typhimurium* 5 days after oral inoculation of BALB/c mice. This result is essentially identical to ours. A nonreplicating $AroA^-$ *S. typhimurium* strain was not increased in virulence in TNF-α-depleted mice, leading to the conclusion that TNF-α suppressed the growth of salmonellae in mice. By using i.v. inoculation of *S. typhimurium* into Ity^r mice, Mastroeni et al. found that splenic and hepatic infections were greatly increased at 5 to 7 days postinoculation by neutralization of TNF-α (25) and that later administration of an anti-TNF-α antibody caused a reinitiation of systemic infection from the established plateau phase (28). We examined the mechanisms by which depletion of IFN-γ and TNF-α caused increased infection by *S. typhimurium* in the experiments described below.

An interesting result of our study was that lack of the virulence plasmid prevented χ3337 from increasing its splenic infection in cytokine-depleted mice compared with the wild-type strain, χ3456. Since neutralization of IFN-γ or TNF-α did not enable χ3337 to behave more similarly to χ3456, the Spv function is not to overcome suppression of bacterial replication mediated by either cytokine. The lack of increased splenic infection by plasmid-cured *S. typhimurium* in IFN-γ-depleted mice in our study is in contrast to the results of Muotiala (30), who reported that the numbers of hepatic CFU of plasmid-cured *S. typhimurium* and *S. enteritidis* were increased 10^3-fold and 100-fold, respectively, by pretreatment of Ity^s mice with an anti-IFN-γ monoclonal antibody. The major experimental difference that could explain our contrasting results is that Muotiala used i.v. inoculation, as opposed to our oral inoculation of salmonellae. We believe that i.v. inoculation of mice with broth-grown salmonellae enables the infection of different sets of host cells in the spleen and/or liver, relative to oral inoculation (19a).

Histopathology of infection in cytokine-deleted mice. To gain some understanding of how the neutralization of cytokines might have affected the host response to wild-type salmonella infection, we examined the histopathology of spleens from mice infected with *S. typhimurium* χ3456 (Fig. 2). A normal spleen section is shown in Fig. 2A with intact white pulp, a well-defined marginal zone, and intact red pulp. Infection with wild-type χ3456 yielded a mean of $10^{4.6}$ CFU per spleen and resulted in increased numbers of large mononuclear cells in the white pulp (i.e., tingible-body macrophages), moderate necrosis of the red pulp including nuclear dust, and moderate inflammation and congestion of the red pulp (Fig. 2B). Depletion of IFN-γ resulted in a nearly 1,000-fold increase in the number of splenic CFU with extensive histopathology (Fig. 2C). Extensive regions of caseating necrosis were evident in both the red and white pulps, the marginal zone was destroyed, and extensive inflammation was observed in both the red and white pulps. Multinucleate giant cells were visible in the red pulp along with increased nuclear dust and congestion (not shown). In contrast, neutralization of TNF-α resulted in a histopathological picture (Fig. 2D) much milder than that of control antibody-treated, infected mice. Although there were increased large mononuclear cells in the white pulp, the amount of necrosis was not as large as would have been expected given the 100-fold-increased splenic CFU compared with control antibody-treated mice. Identical results were obtained with another set of infected mice similarly depleted of IFN-γ or TNF-α.

Mastroeni et al. (26) recently reported that suppression of net salmonella replication after i.v. inoculation of Ity^r and Ity^s mice coincided with granuloma formation in the spleen and liver. Neutralization of TNF-α 3 days after i.v. inoculation increased the number of bacterial CFU and decreased the monocytic responses. They concluded that TNF-α is most likely involved with recruitment of phagocytes, as opposed to stimulation of the phagocytes to kill the bacteria, and that TNF-α is not involved in histopathology. Our results differ from those of Mastroeni et al. (26) in that neutralization of TNF-α in Ity^s BALB/c mice inoculated orally with *S. typhimurium* caused less necrosis in infected spleens than expected

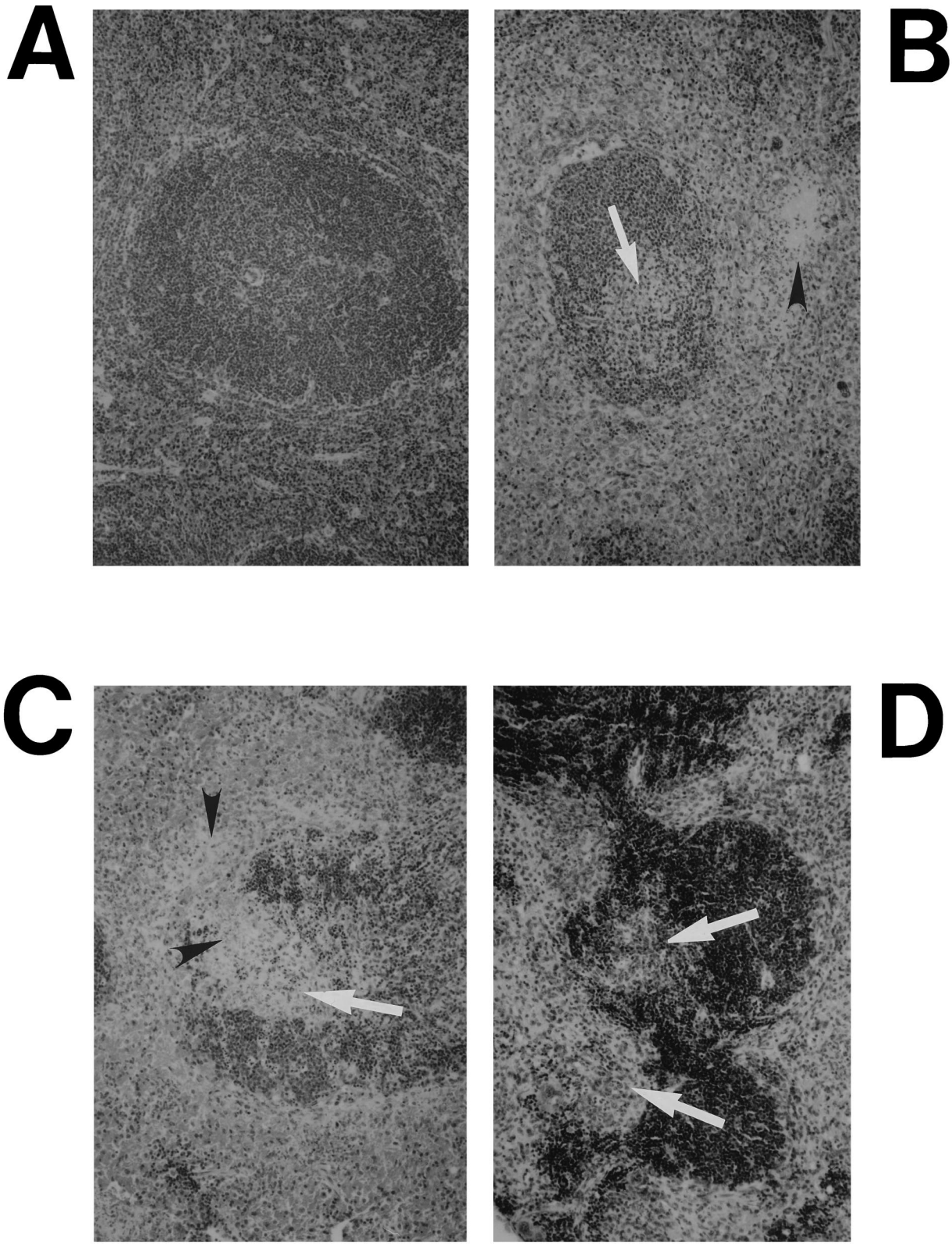

FIG. 2. Splenic histopathology of mice infected with wild-type *S. typhimurium* after treatment with neutralizing antibodies to cytokines. Four mice each were injected i.p. with an anti-IFN-γ or anti-TNF-α monoclonal antibody or an isotype-matched control monoclonal antibody and then inoculated orally with wild-type *S. typhimurium* χ3456. Five days later, a portion of each spleen was homogenized and plated to enumerate the CFU, and the remainder of the tissue was fixed in 10% buffered formalin, embedded in paraffin, divided into 5-μm sections, mounted, and stained with hematoxylin-eosin. Representative tissue of one mouse from each group is depicted as follows: A, normal (uninfected) mouse spleen; B, control monoclonal antibody-treated, infected mouse; C, anti-IFN-γ monoclonal antibody-treated, infected mouse; D, anti-TNF-α monoclonal antibody-treated, infected mouse. White arrows show areas of increased large mononuclear cells in the white pulp; black arrowheads show areas of necrosis. Magnification, ×200.

TABLE 2. Effects of neutralizing IFN-γ and TNF-α on replication versus killing of Spv$^+$ and Spv$^-$ *S. typhimurium* in mice[a]

Antibody treatment	Wild-type *spv* genotype			Δ*spv*::*tet*		
	Total no. of CFU	No. of CFU with pHSG422	Proportion with pHSG422	Total no. of CFU	No. of CFU with pHSG422	Proportion with pHSG422
Control	5.1 ± 0.6	3.0 ± 0.5	−2.1 ± 0.5	3.5 ± 0.8 (<0.001)[b]	2.4 ± 0.9 (NS)[b]	−1.2 ± 0.7 (0.01)[b]
Anti-IFN-γ	7.7 ± 1.0 (<0.001)[c]	5.3 ± 1.0 (<0.001)[c]	−2.4 ± 0.4 (NS)[c]	3.9 ± 0.6 (NS)[c]	2.8 ± 0.9 (NS)[c]	−1.1 ± 0.5 (NS)[c]
Anti-TNF-α	6.8 ± 0.8 (<0.001)[c]	3.4 ± 1.1 (NS)[c]	−3.4 ± 0.8 (<0.005)[c]	4.4 ± 0.8 (<0.05)[c]	3.3 ± 0.8 (0.05)[c]	−1.2 ± 0.6 (NS)[c]

[a] Mice were injected i.p. with monoclonal antibodies on the day of oral inoculation with either wild-type *S. typhimurium* χ3456 or Δ*spv*::*tet S. typhimurium* UF110 containing the marker plasmid pHSG422. Four or 5 days later, total splenic CFU, CFU containing pHSG422, and the proportion of salmonellae still carrying pHSG422 were measured. The proportion of bacteria maintaining pHSG422 is a measurement of the growth rate, while the number of bacteria with pHSG422 is a measurement of killing by the host. All values are $\log_{10}$ (mean ± standard deviation). Combined results of two experiments are shown. There were eight mice in all groups except the anti-TNF-α–wild-type group (there were seven mice in that group due to one death). NS, no significant difference.

[b] *P* value compared with wild-type strain.

[c] *P* value compared with corresponding control antibody-treated mice.

given the elevated numbers of bacteria in the organs. A decreased amount of histopathology in TNF-α-depleted mice is consistent with a major role for TNF-α in endotoxin-mediated damage as a result of salmonella infection (2, 41).

Comparison of splenic infections with wild-type and Δ*spv S. typhimurium* in mice depleted of IFN-γ or TNF-α. During the course of these studies, we developed an *S. typhimurium* strain, UF110 (Δ*spv*::*tet*), for use as a more isogenic Spv$^-$ strain relative to the wild-type genotype, as opposed to virulence plasmid-cured strain χ3337 (15). UF110 contains all of the genes encoded on the virulence plasmid except the *spv* genes, which were replaced with a *tet* cartridge. Hence, if any non-*spv* genes are involved in pathogenesis, they are still present in the Δ*spv* background. We (19) and others (4, 12) have used plasmid pHSG422 to measure the growth and death rates of *S. typhimurium* in infected mice. To elucidate the effects of neutralizing IFN-γ and TNF-α on growth versus killing of salmonellae in mice, we transformed pHSG422 into wild-type *S. typhimurium* χ3456 and Δ*spv*::*tet* UF110. Mice were treated with the same monoclonal antibodies as above and were orally inoculated with either χ3456(pHSG422) or UF110(pHSG422). Four or five days later, splenic CFU were enumerated for the total number of bacteria, the number of bacteria maintaining pHSG422, and the proportion of bacteria maintaining pHSG422 (Table 2).

In the spleens of control-treated mice, there was a significantly higher number of wild-type than Spv$^-$ salmonellae ($P < 0.001$). As we have reported previously (19), the numbers of bacteria maintaining pHSG422 did not significantly differ between the Spv$^+$ and Spv$^-$ strains. The *spv* genotype, therefore, did not significantly affect killing of the bacteria by the mice or movement of the bacteria through mouse tissues (Fig. 1). However, the proportion of wild-type χ3456 with pHSG422 (1 of 80) was significantly lower than in Spv$^-$ strain UF110 (1 of 8). This result indicates that the wild-type strain had undergone more cell divisions over the same period of infection in mice at the nonpermissive temperature for pHSG422, thereby generating more pHSG422-negative bacterial cells. This result confirmed that the growth rate of χ3456 in mice was higher than that of an Spv$^-$ strain, except that the present experiment involved Δ*spv* strain UF110. The splenic recoveries of wild-type χ3456 were increased approximately 400- and 50-fold in mice depleted of IFN-γ and TNF-α, respectively, compared with control mice. In contrast, Spv$^-$ strain UF110 was not significantly increased in IFN-γ-depleted mice and was increased only eightfold in TNF-α-depleted mice ($P < 0.05$). The increased recovery of the Spv$^-$ strain in TNF-α-depleted mice was slightly higher than from the previous experiment using plasmid-cured χ3337. However, it is clear that Spv$^+$ *S. typhimurium* was vastly more proficient than the Spv$^-$ strain at increasing splenic infection in mice depleted of IFN-γ or TNF-α.

To determine how depletion of either IFN-γ or TNF-α resulted in increased splenic infection by wild-type *S. typhimurium*, we examined the carriage of pHSG422. In IFN-γ-depleted mice, the number of χ3456 cells maintaining pHSG422 after 5 days of infection was 200-fold higher than in control-treated mice; however, the proportion of χ3456 with pHSG422 was not significantly different. These results indicate that depletion of IFN-γ resulted in less killing of wild-type *S. typhimurium* by the mice, as opposed to increasing the replication rate of the bacteria. However, the increased number of pHSG422-carrying strain χ3456 bacteria could also mean that the bacteria moved to the spleen in higher numbers (19). In contrast, in TNF-α-depleted mice, the number of strain χ3456 bacteria with pHSG422 was not significantly different from that in controls, while the proportion of χ3456 bacteria with pHSG422 was significantly lower than in controls. These data indicate that depletion of TNF-α caused the more rapid replication of wild-type χ3456. The slightly increased recovery of Spv$^-$ strain UF110 in TNF-α-depleted mice was correlated with a marginally significant increase in the number of UF110 bacteria with pHSG422 ($P = 0.05$) and not with a decrease in the proportion of bacteria maintaining pHSG422.

As noted above, most others concluded that both IFN-γ and TNF-α were responsible for suppression of salmonella replication in mice (28, 31, 32, 40). These conclusions were based primarily on the fact that neutralization of the cytokines did not affect the net recovery of nonreplicating AroA$^-$ or temperature-sensitive *S. typhimurium*. Since nonreplicating salmonellae should be susceptible to killing by the host, it was reasoned that if the cytokines were involved in killing of salmonellae, then neutralization of the cytokines would have increased the recovery of the replication-defective strains. However, there may not be significant killing of the AroA$^-$ strain, even in normal mice (4), or the nonreplicating bacteria may be relatively resistant to bactericidal mechanisms of the host, much the same as nonreplicating bacteria are resistant to the action of certain antibiotics (9, 23). Finally, examining our results in the light of those of others is complicated by the fact that *Ity*^{r} versus *Ity*^{s} mice and different routes of inoculation were used in the different studies. Studies of the effects of the mouse *Ity* genotype on production of IFN-γ in relation to salmonella infection have not always reached similar conclusions (3, 11, 35, 36).

Comparison of splenic infections with wild-type and Δ*spv S. typhimurium* in mice depleted of both IFN-γ and TNF-α. Combined depletion of IFN-γ and TNF-α can result in synergistic

TABLE 3. Effects of combined neutralization of IFN-γ and TNF-α on replication versus killing of Spv^+ and Spv^- *S. typhimurium* in mice[a]

Antibody treatment	Wild-type *spv* genotype			Δ*spv*::*tet*		
	Total no. of CFU	No. of CFU with pHSG422	Proportion with pHSG422	Total no. of CFU	No. of CFU with pHSG422	Proportion with pHSG422
Control	4.8 ± 0.3	2.8 ± 0.7	−1.9 ± 0.7	2.8 ± 0.8 (<0.005)[b]	<1.6 ± 1.0[c] (NS)[b]	<−1.2 ± 0.6 (NS)[b]
Anti-IFN-γ	7.5 ± 0.4 (<0.001)[d]	6.0 ± 0.7 (<0.001)[d]	−1.6 ± 0.5 (NS)[d]	3.8 ± 0.3 (NS)[d]	2.9 ± 0.6 (NS)[d]	−0.9 ± 0.6 (NS)[d]
Anti-TNF-α						

[a] Mice were injected i.p. with monoclonal antibodies on the day of oral inoculation with either wild-type *S. typhimurium* χ3456 or Δ*spv*::*tet S. typhimurium* UF110 containing the marker plasmid pHSG422. Four days later, total splenic CFU, CFU containing pHSG422, and the proportion of salmonellae still carrying pHSG422 were measured. The proportion of bacteria maintaining pHSG422 is a measurement of the growth rate, while the number of bacteria with pHSG422 is a measurement of killing by the host. All values are $\log_{10}$ (mean ± standard deviation). There were four mice per group. NS, no significant difference.

[b] *P* value compared with wild-type strain.

[c] Some mice had no detectable pHSG422-containing salmonellae in their spleens and were assigned the minimum detectable sensitivity for this experiment of 2 CFU/spleen.

[d] *P* value compared with corresponding control antibody-treated mice.

or even qualitatively different effects on infectious processes in mice, as opposed to depletion of the cytokines individually (21, 33). We therefore treated two sets of mice with combined anti-IFN-γ and anti-TNF-α monoclonal antibodies or the control monoclonal antibody and infected the mice with either χ3456(pHSG422) or UF110(pHSG422). With the combined neutralization, wild-type *S. typhimurium* was increased approximately 500-fold ($P < 0.001$) in spleens 4 days postinoculation, whereas Spv^- strain UF110 was increased only 10-fold (not a significant difference) (Table 3). The effect of double neutralization seemed to be to decrease the killing of wild-type salmonellae, as opposed to increasing the growth rate, since the number of χ3456 bacteria with pHSG422 was significantly increased, while the proportion of bacteria with pHSG422 was not significantly changed. This is more similar to the action of IFN-γ depletion than that of TNF-α depletion. The combined cytokine neutralization did not significantly affect the number of UF110 bacteria or the proportion of UF110 bacteria carrying pHSG422. However, the low numbers of UF110 bacteria recovered from spleens in this 4-day infection made analysis of data for pHSG422 difficult with this strain.

Conclusions. In summary, our studies have confirmed that IFN-γ and TNF-α are essential components of the host response for inhibiting systemic infection by *S. typhimurium* in the early phase of disease, before specific immunity has been stimulated. Our data are consistent with the ideas that IFN-γ is involved with increased killing of wild-type salmonellae and that TNF-α is involved with suppression of bacterial replication. Neither cytokine is directly or indirectly responsible for suppressing the replication of Spv^- *S. typhimurium* relative to Spv^+ *S. typhimurium* in systemic infection of orally inoculated BALB/c mice. In terms of elucidating the virulence mechanism encoded by the *spv* genes, our results indicate that if the *spv* genes are involved in the evasion of bacteriostatic host defense functions, these functions must be either constitutively expressed or activated proximally to or independently of TNF-α and IFN-γ. TNF-α is produced by macrophages and other cells as a result of bacterial infection or the presence of bacterial components such as lipopolysaccharide (34). TNF-α is an important component of the complex pathway that leads to the production of IFN-γ by NK cells early in the infection process (2, 41). IFN-γ is involved in the stimulation of macrophages to become more antibacterial (2, 13). One of the functions stimulated in macrophages by IFN-γ in combination with TNF-α is the production of nitric oxide, which is involved with antimicrobial action against a diversity of pathogens, including salmonellae (10). Since neutralization of TNF-α and/or IFN-γ failed to increase the recovery of Spv^- *S. typhimurium*, the *spv* genes are most likely not involved in evasion of nitric oxide-mediated suppression of bacterial replication.

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