

An Essential Role for Gamma Interferon in Innate Resistance to *Shigella flexneri* Infection

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Shigella spp. are the major cause of bacillary dysentery worldwide. To identify immune effectors associated with protection of the naive host during infection, the susceptibility to pulmonary *Shigella* infection of each of various mouse strains that have a targeted deletion in a specific aspect of the immune system was evaluated. Our results demonstrate that mice deficient in gamma interferon are 5 orders of magnitude more susceptible to *Shigella* than are wild-type mice, whereas mice deficient in B and T lymphocytes or in T lymphocytes alone exhibit no difference in susceptibility. Significantly lower numbers of shigellae were recovered from immunocompetent compared with gamma-interferon-deficient mice after infection. While immunocompetent mice were able to clear a sublethal *Shigella* inoculum by day 5 postinfection, progressively increasing numbers of shigellae were cultured from the lungs of gamma interferon-deficient mice over the same period. Histopathology of the lungs from immunocompetent mice infected with a sublethal *Shigella* inoculum showed mild inflammatory changes, whereas the lungs from gamma interferon-deficient mice demonstrated progressively worsening acute bronchiolitis with ulceration. Further, the time to death in gamma interferon-deficient mice correlates inversely with the size of the *Shigella* inoculum. To identify the cellular source of gamma interferon, we infected SCID mice, T-cell-receptor-deficient mice, beige mice (a mouse strain deficient in natural killer [NK] cell activity), and mice depleted of NK cells using anti-asialo-GM₁. Each NK cell-deficient mouse strain exhibited a 10-fold-greater susceptibility to *Shigella* infection than immunocompetent mice. To test the protective effects of gamma interferon in vitro, survival of intracellular *Shigella* was examined in primary macrophages from wild-type mice, primary macrophages from gamma interferon-deficient mice, a macrophage cell line, and a fibroblast cell line. Following activation with gamma interferon, each cell type eradicated intracellular *Shigella*, while nonactivated macrophages fostered *Shigella* replication and nonactivated fibroblast cells fostered both *Shigella* replication and intercellular spread. Taken together, these data establish that NK cell-mediated gamma interferon is essential to resistance following primary *Shigella* infection.

Shigellosis is an invasive disease of the human intestinal tract that is responsible for 200 million cases of bacillary dysentery and 650,000 fatalities annually (15). The disease process is thought to involve bacterial translocation across M cells within the gastrointestinal tract, phagocytosis by macrophages with subsequent lysis of the phagosomes, and induction of apoptosis in infected macrophages with release of proinflammatory mediators (12, 26, 30, 31). Inflammation further promotes *Shigella* invasion and intercellular spread between gut epithelial cells, which leads to necrosis (20, 21). These processes are thought to account for the diarrhea and dysentery that occur in the course of shigellosis.

Immune mechanisms necessary for protection against shigellosis are poorly defined due to the lack of a convenient infection model. While both macrophage and epithelial cell lines can be infected with shigellae, the relative contributions of macrophage and epithelial-cell infection to pathogenesis are unclear. It is becoming increasingly evident that the host inflammatory response plays an important role in *Shigella* pathogenesis. Therefore, manipulable models which provide intact immune systems are necessary in order to elucidate the relationship between host cell infection, the inflammatory response following infection, and pathogenesis. Recently, a murine model of intranasal *Shigella* infection has been reported

(16, 28). Following intranasal infection with a lethal dose of *Shigella*, mice die of an acute pneumonitis (28). Virulent shigellae are able to invade bronchial and alveolar epithelium and elicit both acute suppurative infiltrates and epithelial necrosis, which resembles the lesions seen in intestinal shigellosis (28, 29). In lung lavage fluid of intranasally infected mice, tumor necrosis factor alpha and gamma interferon levels peak at 24 h postinfection, suggesting activation of the Th₁ type of T-helper-cell response (28). This is consistent with the intracytoplasmic residence of shigellae within infected cells. Mice inoculated intranasally with sublethal doses of wild-type *Shigella* or candidate *Shigella* vaccine strains show partial protective immunity to future intranasal challenges with a lethal *Shigella* inoculum (16, 28).

The immune mechanisms which mediate resistance in both primary *Shigella* infection and subsequent exposures remain undefined. To determine which components of the immune system confer protection against primary *Shigella* infection in vivo, the susceptibility of each of various strains of mice with a targeted deletion in a specific aspect of the immune system to intranasal *Shigella* infection was examined. Our results show that gamma interferon is required for protection from *Shigella* infection in vivo and that gamma interferon-mediated activation of host cells leads to killing of shigellae in primary macrophages from wild-type and gamma interferon-deficient mice as well as in macrophage- and fibroblast-derived cell lines in vitro.

MATERIALS AND METHODS

Mouse strains. The immunocompetent mouse strains BALB/c and C57BL/6, T-cell-receptor-deficient mice (C57BL/6 *tcrl* × *tcrl* × *tcrl* × *tcrl*), and the natural

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killer (NK) cell-deficient mouse strain beige (*C57BL/6 bg^l/bg^l*) were obtained from Jackson Laboratory (Bar Harbor, Maine). Immunocompetent 129SvEv mice were obtained from Taconic (Germantown, N.Y.). Mice with severe combined immunodeficiency (*BALB/c scid/scid*) were obtained from Harris Goldstein. Major histocompatibility complex class II-deficient ($A\beta^{0-/-}$), major histocompatibility complex class I-deficient (homozygously negative for β_2 -microglobulin [$\beta_2M^{-/-}$]) and gamma interferon knockout ($GKO^{-/-}$) mice, which were constructed in the *C57BL/6* \times 129SvEv background and had been backcrossed several times onto the *C57BL/6* background, were obtained from Barry R. Bloom. To confirm that the mixed-strain background of these mice would not confound our results, we examined the susceptibilities of the stem cell parental 129SvEv mouse strain (Taconic), homozygous $GKO^{+/+}$ littermates of $GKO^{-/-}$ mice that had been maintained by sibling matings, and gamma interferon-deficient mice on the *C57BL/6* genetic background (Jackson Laboratory) as described in Results.

Mice were between 5 and 7 weeks of age at the time of infection and were sex matched between groups. Mice were housed in a pathogen-free environment at the Albert Einstein College of Medicine. Care of animals was in accordance with guidelines set by the American Association for Accreditation of Laboratory Animal Care and the Albert Einstein College of Medicine.

Bacteria. The wild-type *Shigella flexneri* serotype 2a strain 2457T and its virulence plasmid-cured derivative, BS103, have been described (17). Strains were routinely grown in tryptic soy broth.

Cytokines. Recombinant murine, rat, and human gamma interferons were obtained from Gibco BRL (Grand Island, N.Y.).

Mouse infections. For mouse infections, *S. flexneri* was subcultured from an overnight culture to mid-exponential phase (optical density at 600 nm, 0.5 to 1.0), washed with phosphate-buffered saline (PBS), and adjusted to the appropriate concentration prior to inoculation. Numbers of bacteria per inoculum were confirmed by plating serial dilutions of the inoculum. To determine the lethal dose of *Shigella*, groups of 4 to 10 mice of each strain were inoculated intranasally with serial 10-fold dilutions of the wild-type *S. flexneri* serotype 2a strain 2457T as described previously (28). Briefly, mice were anesthetized intramuscularly with a mixture of 0.6 mg of ketamine hydrochloride (Ketaset; Aveco Co., Fort Dodge, Iowa) and 0.18 mg of xylazine hydrochloride (Rompun; Mobay Corp., Shawnee, Kans.) in 100 μ l of saline. An inoculum of *Shigella* resuspended in 30 μ l of saline was then introduced dropwise into the nares. Mice were examined 2 to 4 h after infection to confirm recovery from the anesthetic and were monitored daily thereafter for survival. Saline controls were included in all experiments to control for mortality and morbidity that might occur as a result of the experimental technique. To quantitate the effects of gamma interferon on *Shigella* eradication in vivo, *C57BL/6* and $GKO^{-/-}$ mice were infected with 10^5 CFU of 2457T. At days 1, 3, and 5 postinfection, lungs from these mice were surgically removed and homogenized. Dilutions of lung lysates were plated onto tryptic soy agar containing Congo red (0.01%).

NK cell depletion. In vivo depletion of NK cells was achieved by intravenous injection of 0.2 ml of PBS containing 40 μ l (39 μ g/ml) of rabbit anti-asialo-GM1 antiserum (Wako BioProducts) at 2 days prior to infection and at 1 and 4 days postinfection as described by the manufacturer. To verify that differences in susceptibilities observed following NK cell depletion were not due to nonspecific effects of rabbit antibody, intravenous injection of 0.2 ml of PBS containing 40 μ l (40 μ g/ml) of rabbit immunoglobulin G (Sigma Chemical Co.) was used as a control.

Cell lines. Primary bone marrow-derived macrophages were harvested from the femurs of $GKO^{-/-}$ and *C57BL/6* mice and cultured in Dulbecco's modified Eagle medium supplemented with 20% fetal calf serum (FCS) and 30% L929 cell supernatant for at least 7 days prior to infection. Mouse macrophage J774 cells, rat lung fibroblast L2 cells, human cervical epithelioid carcinoma HeLa cells, and mouse areolar connective tissue L929 cells (L cells) were obtained from the American Type Culture Collection. J774 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% FCS and 5% NCTC-109. L2, HeLa, and L cells were maintained in minimal essential medium (MEM) supplemented with 10% FCS and 1 \times nonessential amino acids.

In vitro infections. For infections of all cell lines, shigellae, diluted in MEM, were centrifuged (700 \times g) onto monolayers of adherent cells within tissue culture dishes for 10 min at 30°C. For infection of primary bone marrow macrophages, 2.4×10^5 CFU of 2457T were used to infect 2.2×10^5 to 2.4×10^5 cells seeded in 48-well tissue culture plates 12 h prior to infection. For infection of J774 cells, 1.25×10^6 to 1.50×10^6 CFU of 2457T were used to infect a confluent 35-mm-diameter petri dish of cells. At 15 min postcentrifugation, the bacterial suspension was replaced with DMEM supplemented with FCS (10%), NCTC 109 (5%), and gentamicin (50 μ g/ml). Gentamicin kills extracellular but not intracellular shigellae. To quantitate the number of viable intracellular shigellae, adherent cells were washed three times with MEM at the indicated time points and lysed with 0.1% sodium deoxycholate in saline. Serial dilutions of the cell lysate were then plated onto tryptic soy agar containing Congo red (0.01%).

For infection of fibroblast cells, 5.0×10^5 to 7.0×10^5 CFU of 2457T were used to infect a confluent monolayer within a 35-mm petri dish. At 80 min postcentrifugation, the bacterial suspension was replaced with MEM supplemented with FCS (10%) and gentamicin (25 μ g/ml). Quantitation of viable shigellae in fibroblast cell lines was carried out for 15 h postinfection, whereas quantitation in macrophage cells was carried out for only 6 h, since *Shigella*

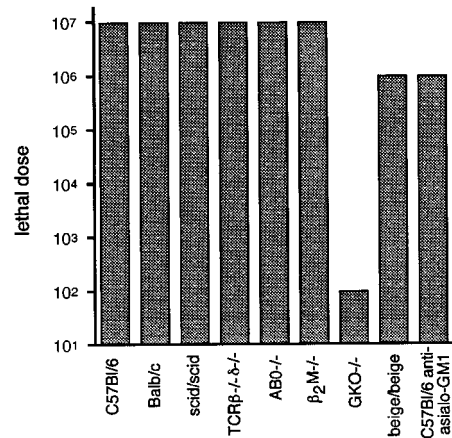


FIG. 1. Susceptibilities of different mouse strains to intranasal *Shigella* infection. Age- and sex-matched groups of 4 to 10 mice were infected with serial 10-fold dilutions of wild-type *S. flexneri* 2457T in saline or with saline alone as described in Materials and Methods. *C57BL/6* anti-asialo-GM1 are mice depleted of NK cells as described in the text. Reported lethal doses represent the *Shigella* inoculum that caused $\geq 90\%$ mortality.

induces apoptosis of macrophages beginning at approximately 3 h postinfection (31); *Shigella* infection is not known to induce apoptosis of fibroblast cells. Assays of *Shigella* plaque formation on fibroblast cell monolayers were performed as previously described (19). Cells were examined for the formation and size of bacterial plaques at 24 and 48 h postinfection.

For experiments involving gamma interferon activation of primary macrophages, as well as macrophage and fibroblast cell lines, species-specific gamma interferon was added to a final concentration of 100 U/ml to the media in which cells were maintained both overnight prior to infection and during all steps of the infection.

Statistics. The statistical significance of data was determined by the independent Student *t* test, with a *P* value of <0.05 being taken as significant.

RESULTS

Susceptibility of different immune knockout mouse strains to primary *Shigella* infection. Mice with targeted deletions in specific aspects of the immune system are valuable tools to study the immune effectors necessary for protection against various infectious agents (4, 10, 13, 14). To assess which immune cells or factors are important in protection following primary *Shigella* infection, the susceptibility of various immune-deficient mouse strains to intranasal infection was determined (Fig. 1). The lethal dose of *S. flexneri* for both *C57BL/6* and *BALB/c* immunocompetent mice is 10^7 CFU, a finding consistent with the previously reported 50% lethal dose of 10^7 CFU for strain 2457T in *BALB/c* mice (16, 28). Further, *Shigella* organisms killed prior to inoculation by exposure to UV light or the virulence-plasmid-cured derivative of 2457T, strain BS103 (17), which is unable to invade nonphagocytic cells, do not cause morbidity in *C57BL/6* mice (data not shown).

Gamma interferon-deficient mice ($GKO^{-/-}$) exhibited susceptibility to *Shigella* infection that was increased by 5 orders of magnitude, whereas no differences in susceptibility were observed in mice that were $\beta_2M^{-/-}$, $A\beta^{0-/-}$, homozygously negative for β and δ T-cell-receptor subunits ($TCR\beta^{-/-}\delta^{-/-}$), or *SCID* compared with *C57BL/6* or *BALB/c* mice (Fig. 1). To confirm that the observed difference in susceptibility of the $GKO^{-/-}$ mice was not due to mouse strain background differences, we examined the susceptibilities of the stem cell parental 129SvEv mouse strain and of the homozygous $GKO^{+/+}$ littermates of $GKO^{-/-}$ mice having the background of the animals used in subsequent studies presented here that had

TABLE 1. Survival of *Shigella* within the lungs of C57BL/6 immunocompetent mice or GKO^{-/-} gamma interferon-deficient mice after infection with 10⁵ CFU of wild-type *Shigella* strain 2457T

Day postinfection	No. of viable shigellae per mouse in strain:		P value
	C57BL/6	GKO ^{-/-}	
1	1.4 × 10 ⁴	1.8 × 10 ⁵	0.0004
3	7.8 × 10 ¹	2.7 × 10 ⁵	0.013
5	4.0 × 10 ⁰	9.6 × 10 ⁵	0.0016

been maintained by sibling matings. Each of these mouse strains has the same susceptibility as C57BL/6 mice (data not shown). Further, gamma interferon-deficient mice crossed extensively into the C57BL/6 genetic background have the same increased susceptibility as GKO^{-/-} mice used in this study.

To determine the contribution of NK cell-produced gamma interferon to protection, we examined the susceptibilities of the NK cell-deficient mouse strain beige and C57BL/6 mice depleted of NK cells by using rabbit anti-asialo-GM₁ antiserum. Each NK cell-deficient mouse strain had a 10-fold-increased susceptibility to *Shigella* infection compared with immunocompetent mice (Fig. 1), whereas no difference in susceptibility was observed in mice treated with rabbit serum immunoglobulin G.

Effect of gamma interferon on *Shigella* survival in vivo. To determine whether differences in susceptibility between mouse strains correlate with the ability of the mice to clear the infection, the number of viable *Shigella* organisms present within the lungs of infected C57BL/6 and GKO^{-/-} mice was quantitated at various time points following infection with 10⁵ CFU of wild-type *Shigella* (Table 1). Significantly increased numbers of viable shigellae were recovered from the lungs of GKO^{-/-} mice as compared with C57BL/6 mice at days 1, 3, and 5 postinfection ($P < 0.05$, GKO^{-/-} versus C57BL/6 mice at each time point). A 3,600-fold decrease was observed in the numbers of shigellae in C57BL/6 mice from day 1 to day 5 postinfection ($P < 0.05$). The progressive fall in the number of shigellae recovered from C57BL/6 mice, beginning at inoculation and continuing over time, indicates that in the presence of gamma interferon, killing begins upon infection and clearance of the infection is achieved by day 5 postinfection for a *Shigella* inoculum 2 logs below the lethal dose. Following infection with the same dose, there was a fivefold increase in the number of shigellae recovered from the lungs of GKO^{-/-} mice ($P < 0.05$) within the same period. These data indicate that the role of gamma interferon in mediating protection from *Shigella* infection is in the promotion of *Shigella* clearance. Since a significant percentage of GKO^{-/-} mice infected with 10⁵ CFU of 2457T die by day 5 postinfection (see Fig. 3), GKO^{-/-} mice that survived to day 5 are more likely to be better able to survive; thus, the number of viable shigellae isolated from the lungs of these animals probably represents an underestimate of the number that theoretically would have been seen at this time point.

Histopathology of infected gamma interferon-deficient and wild-type mice. The increased susceptibility of GKO^{-/-} versus C57BL/6 mice to intranasal *Shigella* infection is supported by histopathologic examination. After infection with 10⁵ organisms, GKO^{-/-} mice show early acute bronchiolitis at day 1 postinfection (Fig. 2A), followed by multifocal mucosal ulceration and intense mixed inflammatory infiltrate by day 3 (Fig. 2C). This progresses to nearly complete mucosal ulceration with exuberant acute neutrophilic exudate filling airway lumina

by day 5 (Fig. 2E). In contrast, C57BL/6 animals infected with the same-size *Shigella* inoculum show a mild peribronchiolar and interstitial inflammatory infiltrate without mucosal ulceration beginning at day 1 postinfection (Fig. 2B). By day 3 postinfection, the chronic inflammation is mildly increased (Fig. 2D). This inflammation is completely resolved by day 5 (Fig. 2F). The histopathology observed in GKO^{-/-} mice following intranasal *Shigella* infection with 10⁵ organisms shows a progression that is similar to histopathologic changes previously observed in immunocompetent mice following administration of a lethal dose of *Shigella* (28).

Time to death in gamma interferon-deficient mice following infection with various *Shigella* inocula. To determine whether lethality as determined by the time to death correlates with size of the *Shigella* inoculum, we examined the time to death in gamma interferon-deficient mice following infection with 10-fold increases in bacterial inoculum above the lethal dose (Fig. 3). The time to death in gamma interferon-deficient mice was observed to correlate inversely with the *Shigella* inoculum size. The average time to death following infection with 10⁶ CFU of *Shigella* was 3.3 ± 1.2 (mean ± standard deviation) days compared with 21.3 ± 10.8 days after infection with 10² CFU ($P < 0.05$). These data suggest that lethality in mice caused by *Shigella* is dose dependent.

Effect of gamma interferon activation on *Shigella* intracellular survival in bone marrow-derived macrophages from GKO^{-/-} and C57BL/6 mice. To determine whether the in vivo difference observed between GKO^{-/-} and C57BL/6 mice correlates with the effects of gamma interferon activation of primary macrophages obtained from these mice in vitro, the effects of gamma interferon pretreatment on the intracellular viability of *Shigella* in bone marrow-derived macrophages was evaluated. Beginning at 2.5 h postinfection and at all time points observed thereafter, significant differences in intracellular *Shigella* survival were observed between gamma interferon-activated and nonactivated macrophages derived from either C57BL/6 or GKO^{-/-} mice (Fig. 4A). No significant differences in *Shigella* survival were observed in macrophages from C57BL/6 and GKO^{-/-} mice under either condition.

Effect of gamma interferon activation of J774 cells on intracellular *Shigella* survival. To further evaluate the effects of gamma interferon activation of cells in vitro, the intracellular viability of *Shigella* in the J774 macrophage cell line was examined (Fig. 4B). Beginning at the first hour postinfection and at all time points observed thereafter, significantly lower numbers of intracellular shigellae were recovered from cells activated with gamma interferon as compared with cells not activated. In nonactivated cells, a significant (twofold) increase ($P < 0.05$) in the numbers of intracellular shigellae was observed between 3 and 5 h postinfection, demonstrating the ability of *Shigella* to replicate within these cells.

Effects of gamma interferon activation of fibroblast cells on *Shigella* plaque formation and intracellular survival. The ability to spread from cell to cell is critical to *Shigella* pathogenesis (3, 27). The intercellular spread of *Shigella* can be assessed by its ability to form plaques on confluent monolayers of cells in tissue culture. To evaluate the effects of gamma interferon activation of cells on their ability to foster *Shigella* intercellular spread, confluent monolayers of L2, HeLa, and L cells were pretreated with recombinant rat, human, and mouse gamma interferon, respectively, and assessed for bacterial plaque formation following *Shigella* infection. In all cell lines tested, no bacterial plaques were formed in cells activated with species-specific gamma interferon, whereas bacterial plaques were consistently formed in cells either not activated with gamma

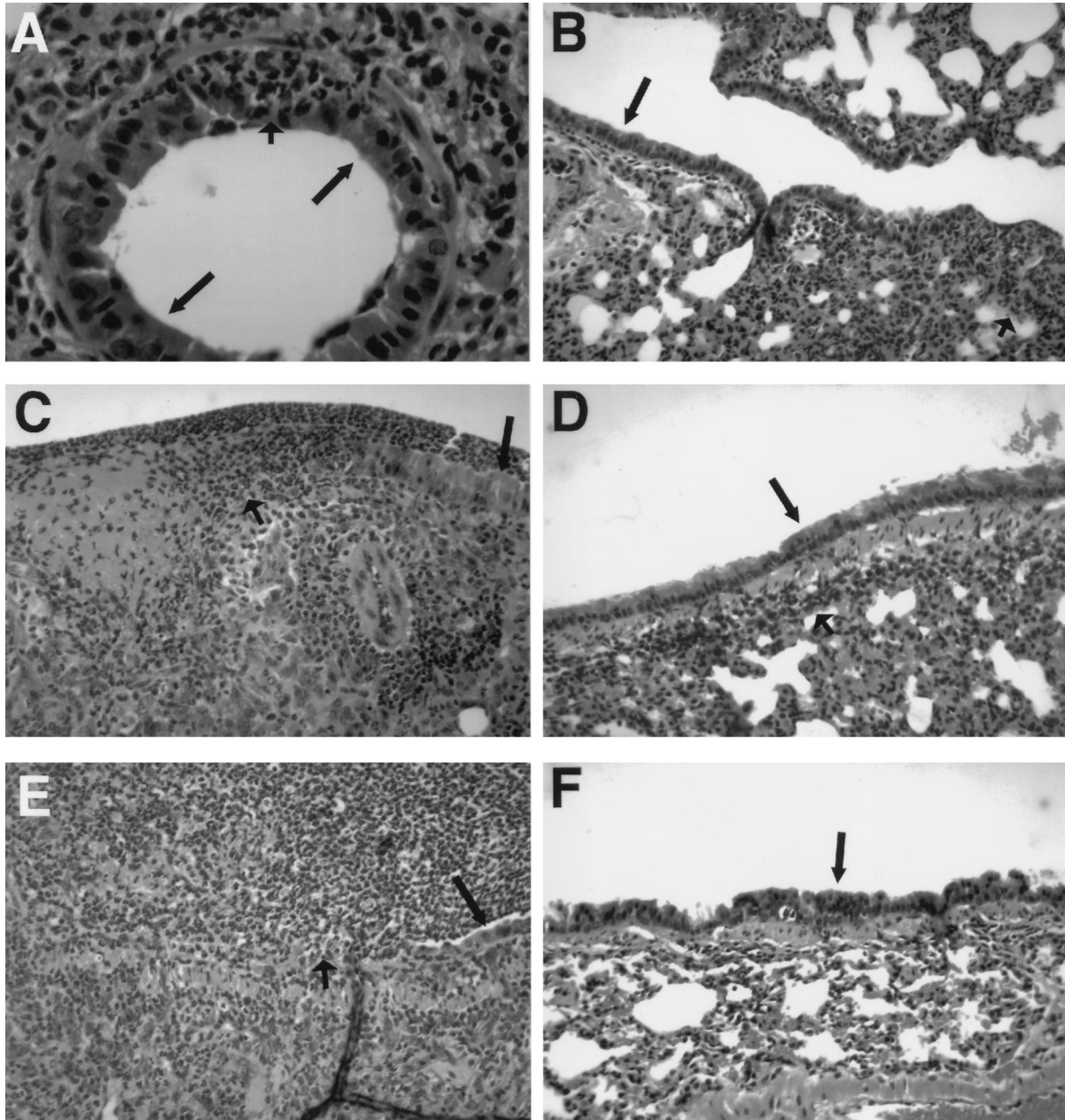


FIG. 2. Hematoxylin-and-eosin-stained sections of lung from GKO^{-/-} (A, C, and E) and C57BL/6 (B, D, and F) mice after infection with a 10^5 -CFU inoculum of *S. flexneri*. (A) On day 1, bronchioles show early acute inflammation (short arrow) with no disruption in bronchiolar epithelium (long arrows). (C) On day 3, bronchiolar epithelium (long arrow) shows ulceration (short arrow) with polymorphonuclear cell exudate, fibrin, and intense acute and chronic inflammation in the bronchiolar wall. (E) On day 5, near-complete epithelial destruction is evident with residual epithelium (long arrow). The luminal space is filled with polymorphonuclear cells, with obstruction of the airway (short arrow). (B) On day 1, a longitudinal section of terminal bronchiole shows absence of acute bronchiolitis. A mild acute and chronic lymphocytic pneumonitis is present (long arrow, bronchiolar epithelium). (D) On day 3, mild peribronchiolar chronic lymphocytic inflammation (short arrow) is seen adjacent to intact bronchiolar epithelium (long arrow). (F) On day 5, inflammatory infiltrate is resolved (long arrow, bronchiolar epithelium). Magnifications: $\times 80$ (A) and $\times 30$ (B to F).

interferon (Fig. 5) or activated with gamma interferon from another species (data not shown).

To determine whether the inability of *Shigella* to form plaques in gamma interferon-pretreated cells is due to decreased cellular invasion or to increased intracellular *Shigella* killing, we quantitated the numbers of viable intracellular shigellae from infected L2-cell monolayers that had been pretreated or not pretreated with gamma interferon. Beginning at 6 h postinfection and at all time points observed thereafter,

significantly lower numbers of shigellae were recovered from cell monolayers activated with gamma interferon as compared with cells not activated (Fig. 4C). Notably, there were no significant differences in the numbers of recoverable shigellae at any time point prior to 6 h postinfection (Fig. 4C). Thus, gamma interferon activation of L2 fibroblast cells leads to increased killing of intracellular shigellae.

To confirm that the decreased numbers of intracellular *Shigella* in gamma interferon-activated cells is not due to the effect

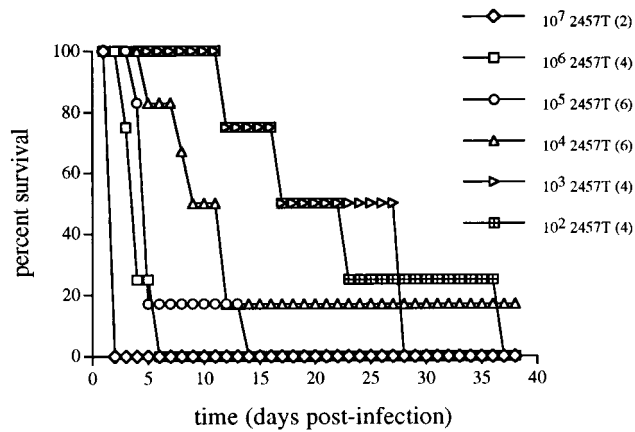


FIG. 3. Time to death in gamma interferon-deficient mice following infection with a lethal dose of *S. flexneri* wild-type strain 2457T (10^2 CFU) and serial 10-fold increases above the lethal dose. The number of mice infected per dose is indicated in parentheses.

of decreased plasma membrane integrity or increased cell death, we examined the ability of gamma interferon-activated cells to exclude the dye trypan blue at 15, 24, and 48 h postinfection. At all time points, gamma interferon-activated cells demonstrated trypan blue exclusion to the same extent as uninfected monolayers or monolayers not activated with gamma interferon (data not shown).

DISCUSSION

This study establishes the essential requirement for gamma interferon in host protection against primary *Shigella* infections. Immune knockout mice lacking this cytokine exhibit an increase of 5 orders of magnitude in susceptibility to *Shigella* infection compared with mice not lacking this cytokine. The deleterious effects of gamma interferon deficiency could be counteracted in vitro by providing exogenous gamma interferon to infected primary macrophages from either wild-type or gamma interferon-deficient mice or to infected macrophage or fibroblast cell lines. Following infection, significant *Shigella* replication occurs in each of these cell types in the absence of gamma interferon, as well as in the lungs of gamma interferon-deficient mice. In contrast, isolated primary macrophages and macrophage and fibroblast cell lines activated with gamma interferon are able to promote killing of *Shigella*. In a similar fashion, *Shigella* is readily cleared from the lungs of immunocompetent mice and is not cleared from the lungs of gamma interferon-deficient mice. These data, taken together with the observation that the time to death following infection in gamma interferon-deficient mice decreases with increasing doses of *Shigella*, support the requirement for *Shigella* replication in pathogenesis and establish that the protective role of gamma interferon is to promote clearance of intracellular *Shigella*.

Consistent with these results are previously reported data that have suggested that gamma interferon plays a protective role following *Shigella* infection. Examination of rectal biopsy specimens has demonstrated increased levels of gamma interferon cytokine and mRNA, as well as increased expression of the gamma interferon receptor, in shigellosis patients compared with healthy controls. In shigellosis patients, the expression of gamma interferon is twofold higher during convalescence than during the acute stage (22, 23).

The two known cellular sources of gamma interferon are T

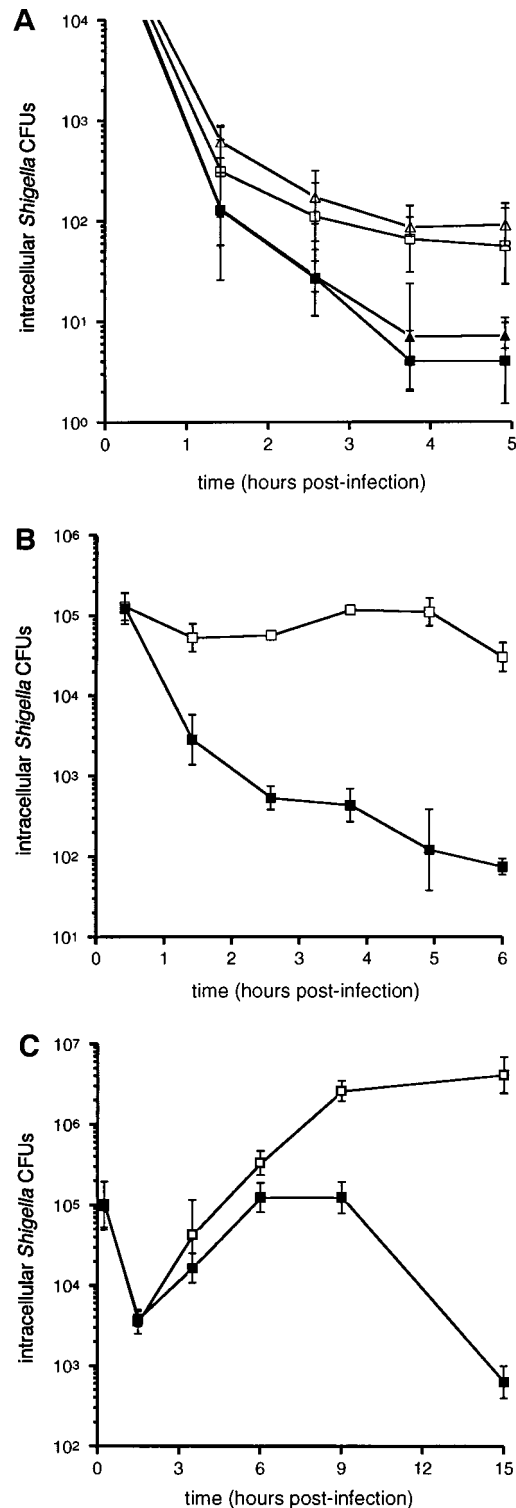


FIG. 4. (A) Intracellular survival of *Shigella* in bone marrow-derived macrophages from C57BL/6 (squares) and $GKO^{-/-}$ mice (triangles). Cells were either not activated (open symbols) or activated (solid symbols) with 100 U of gamma interferon per ml. Each datum point represents the mean of six independent determinations. (B) Intracellular survival of *Shigella* within J774 macrophagelike cells with (solid squares) or without (open squares) activation with gamma interferon (100 U/ml). Each datum point represents the mean of five independent determinations. (C) Intracellular survival of *Shigella* within L2 cells with (solid squares) or without (open squares) activation with gamma interferon (100 U/ml). Each datum point represents the mean of four independent determinations. Bars indicate one standard deviation.

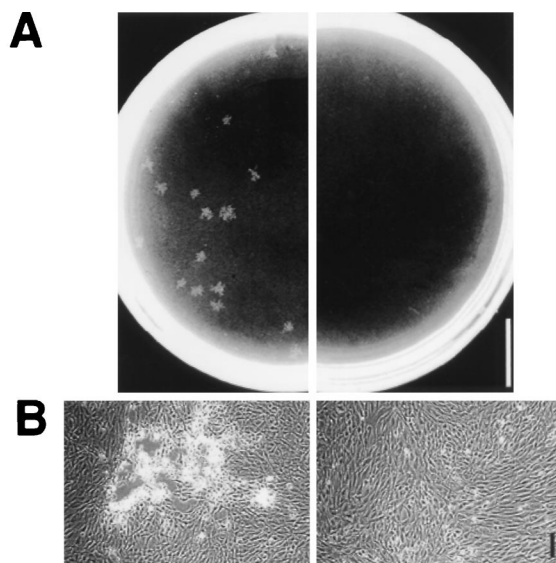


FIG. 5. Effects of gamma interferon activation on the ability of *Shigella* to spread from cell to cell. Bacterial plaque formation in L2 cell monolayers at 48 h postinfection with (right panels) or without (left panels) activation by 100 U of gamma interferon per ml. Bars: 1 cm (A) and 100 μm (B).

cells and NK cells (1, 7). In the present study, mice with complete T-cell deficiency ($\text{TCR}\beta^{-/-}\delta^{-/-}$ and SCID), deficiency in specific subsets of T cells ($\beta_2\text{M}^{-/-}$ and $\text{A}\beta^{-/-}$), or NK cell deficiency (beige), as well as C57BL/6 mice depleted of NK cells by using anti-asialo- GM_1 , were examined to determine the cellular source of gamma interferon necessary for resistance. Our data suggest that NK cells are the major source of gamma interferon following primary *Shigella* infection, since a 10-fold increase in susceptibility was observed in both the NK cell-deficient mouse strain (beige) and the NK cell-depleted mice relative to the wild-type mice, whereas no difference in susceptibility was observed in T-cell-deficient mice. These findings are consistent with previous data demonstrating NK cells to be the major source of gamma interferon following primary infection with another intracellular pathogen, *Listeria monocytogenes* (1, 7). Depletion of NK cells in vivo using both monoclonal and polyclonal antibodies to NK cell antigens led to exacerbation of *Listeria* infection, whereas depletion of T cells had no effect (7). In these studies, as in the present study, the observation that gamma interferon-depleted mice are more susceptible to bacterial infection than wild-type mice experimentally depleted of NK cells suggests that antibody depletion of NK cells is incomplete (7). In the present study, mice with NK cell deficiency (beige) demonstrate the same increased susceptibility to *Shigella* infection as mice experimentally depleted of NK cells. The observation that beige mice are more resistant to *Shigella* infection than gamma interferon-deficient mice is likely a result of the previously observed leakiness in NK cell deficiency in the beige mouse (24, 25).

In this study, the essential role of gamma interferon in the clearance of intracellular *Shigella* from bone marrow-derived mouse macrophages, a mouse macrophage cell line, and human, rat, and mouse fibroblast cell lines is definitively demonstrated. The role of gamma interferon in the activation of macrophages to kill other intracellular pathogens has been described extensively (2, 8, 9); the induction of reactive oxygen and nitrogen intermediates is felt to mediate this effect (6). In contrast, while the role of gamma interferon in the activation of fibroblast killing of intracellular pathogens has been de-

scribed, the mechanism of this effect has not been well characterized (5). We have demonstrated that reactive nitrogen intermediates do not mediate this effect in either macrophages or fibroblasts (unpublished data). Further studies will be performed to evaluate the mechanism of gamma interferon induction of *Shigella* killing by macrophage and fibroblast cells.

Two previous studies have suggested that gamma interferon may alter the course of *Shigella* infection in activated HeLa, FS-1, or primary rabbit kidney cells (11, 18). By measuring ^3H incorporation following actinomycin D inhibition of host cell transcription and then counting the number of intracellular bacteria within infected cells, Gober et al. (11) concluded that interferons are able to suppress *Shigella* intracellular growth. However, this study examined the numbers of intracellular *shigellae* only at 7 h postinfection. Since actinomycin D inhibits host cell transcription, signaling by gamma interferon would also be expected to be inhibited, making interpretation of this result difficult. Using an agarose overlay procedure over infected cell monolayers, Niesel et al. have found evidence that treatment with gamma interferon decreases *Shigella* invasiveness (18). In this study, the quantitation of the numbers of focal areas of bacterial infection was performed at 60 min postinfection; thus, the observed reduction in the number of *Shigella* colonies from intracellular bacteria could reflect a decreased intracellular survival of *Shigella* within the first hour postinfection equally well as a decrease in the efficiency of invasion. Our data clearly demonstrate that the efficiency of invasion is not altered by pretreatment of cells with gamma interferon, since there were no significant differences in the numbers of recoverable intracellular *Shigella* at any time point prior to 6 h postinfection, but that *Shigella* intracellular survival in gamma interferon-activated cells is dramatically curtailed at later time points postinfection. These data, taken together with the marked increased susceptibility of gamma interferon-deficient mice compared with that of immunocompetent mice, establish that gamma interferon is essential to host resistance to primary *Shigella* infection.

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