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Mouse Fibroblast Interferon Modifies *Salmonella typhimurium* Infection in Infant Mice

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The effect of mouse fibroblast interferon on *Salmonella typhimurium* infection in infant mice was examined. The lethality to mice that had been given *S. typhimurium* intragastrically was significantly reduced in a dose-dependent manner when the mice were pretreated with fibroblast interferon. Lower doses of interferon delayed the development of disease. Interferon neutralized with anti-interferon globulin did not influence the lethality of *S. typhimurium* to mice. In mice treated with interferon there was also a reduced invasiveness of *S. typhimurium* in intestinal epithelial cells in vivo. It was further demonstrated in an in vitro system that interferon pretreatment of mouse L-929 cells inhibited the invasiveness of the bacteria in a dose-dependent manner. The in vitro inhibition was neutralized with anti-interferon globulin. The results indicate that interferon inhibits *Salmonella* bacteria from invading cells and establishing an intracellular state of infection. This may represent an important factor in the pathogenesis of disease.

Salmonella typhimurium causes a disease in mice that appears to be similar in its pathogenesis to human typhoid fever. One of the major features characterizing bacteria belonging to the *Salmonella* group is the ability of these bacteria to invade epithelial cells in the intestines of mice and humans. For *S. typhimurium* several studies have shown that in vivo pathogenicity is well correlated with the ability to invade HEp-2 and HeLa cell culture monolayers in vitro (3, 11). For the majority of the enteroinvasive bacteria, *Salmonella* spp. (16), *Shigella* spp. (21), and enteroinvasive *Escherichia coli* (13), the in vitro invasiveness in cell cultures seems to be dependent on the presence of a plasmid.

Interferons are frequently present in the host during infections with bacteria and other infectious agents (1, 8, 15, 18, 22). It has been demonstrated that interferon can influence several host defense mechanisms against bacterial infections, e.g., uptake and degradation of bacteria by macrophages and granulocytes (2, 9, 12), and modify the immune response to various infectious agents (22). Other studies have indicated that mouse fibroblast interferon can modify the pulmonary infection of mice infected with *Haemophilus influenzae* (9) and that immune interferon may reduce the mortality of mice infected intraperitoneally by *S. typhimurium* (14). In a previous study we showed that human leukocyte interferon can inhibit the uptake of *S. typhimurium* in HEp-2 cells (3). The effect was dose dependent and could be neutralized by anti-interferon globulin.

To study whether this effect can be reproduced in vivo, an animal model was established. In the present study we examined the effect of mouse fibroblast interferon on infant mice infected experimentally with *S. typhimurium* given intragastrically. The effect of interferon on bacterial invasiveness in small intestine epithelial cells in vivo, as well as the effect of interferon on the invasiveness of *S. typhimurium* in mouse L-929 cells in vitro, was examined.

MATERIALS AND METHODS

Mice. Randomly bred 2- to 3-day-old infant Swiss mice (strain NMRI:Bom) were used. The mice were kept in cages with their mothers until bacterial challenge.

Interferon and anti-interferon globulin. Partially purified mouse fibroblast interferon (alpha plus beta) (2×10^6 U/mg of protein) was obtained from the late K. Paucker, Philadelphia, Pa. Anti-interferon globulin was obtained from A. D. Inglot, Wroclaw, Poland. A 1/10,000 dilution of this preparation neutralized 10 U of interferon. Interferon activity was assayed by the infectivity inhibition microtest (7) by using vesicular stomatitis virus and L-929 cells. One unit of the reference standard preparation G-002-904-511, obtained from the Institute of Allergy and Infectious Diseases, Bethesda, Md., was equivalent to 1.0 U in our system.

Interferon treatment of mice. The interferon preparation was diluted in basal Eagle medium (BME), and 0.2 ml containing 100 to 1,000 U was injected subcutaneously into the mice in the neck area. Control mice were given 0.2 ml of BME.

Preparation and administration of bacteria. *S. typhimurium* SIFF S4575/81 was grown on heart infusion agar for 24 h at 37°C. The cultures were harvested in 0.9% NaCl and suspended to a concentration of ca. 2×10^8/ml as determined spectrophotometrically (Hitachi model 101 spectrophotometer). The concentration of live bacteria was determined by infectivity titration. The bacterial suspension was administered intragastrically by the esophageal route with a soft plastic tube (0.2 ml per mouse). Special care was taken not to damage the esophagus. Control mice that were not infected with bacteria were given 0.2 ml of NaCl through the gastric tube. After the administration of the bacterial suspension, the infant mice were randomly redistributed to the mothers. The mice were kept by their mothers throughout the assay, and the numbers of live and dead mice were recorded at various time periods after inoculation.

Disintegration of bacterial cultures by freeze pressing. *S. typhimurium* cultures (24 h old) from heart infusion agar were harvested in 0.9% NaCl to a suspension of 2.9×10^8 live bacteria per ml. The bacterial cells were disintergrated by means of an X-press (model X-5, AB Biox, Järnfella, Sweden) (10). Altogether the bacterial suspension was pressed five times with 2.5×10^3 kPa of pressure. After the treatment the viable bacterial count was reduced to 6.5×10^5 bacteria per ml.

Detection of *S. typhimurium* in mice. Mice that had been

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treated with interferon and control mice were killed at different times after inoculation with bacteria. The abdomen was opened aseptically, and fine-needle biopsies for bacteriological examination were taken from the liver parenchymal tissue by means of sterile syringe needles. The samples were homogenized and examined for growth after incubation on blood agar plates at 37°C for 24 h.

Ten-millimeter sections of the upper part of the small intestine were prepared as direct smears by squeezing the material between two glass microscopy slides. These samples were fixed for 24 h in 2.0% glutaraldehyde in cacodylate buffer and stained for 4 min in acridin orange (50 mg/liter). The samples were then examined in a combined UV incident-light/differential-interference contrast microscope as described below. Areas of the field where monolayers of epithelial cells could be recognized were examined. The number of bacteria per cell and the number of cells infected were recorded. One hundred cells in each preparation were counted. Parallel preparations from five animals were examined each time.

Cells. Mouse L-929 cells were grown on glass cover slips (14 mm in diameter) in 24-well tissue culture plates (Falcon 3008). Each well was supplied with 1 ml of cell suspension, ca. 10^5 cells in BME containing bicarbonate, 10% fetal bovine serum, and 100 μg of streptomycin and 100 μg of penicillin G per ml. The culture plates were incubated for 24 h at 37°C in a 5% CO_2 atmosphere with saturated humidity. The cell cultures were recognized as suitable for inoculation when they formed an almost continuous monolayer with intermediate blank areas (leopard spots). Before inoculation they were washed three times with 0.15 M phosphate-buffered saline (PBS) (37°C), incubated for 1 h in BME, and then washed three times again with PBS to minimize residual traces of antibiotics. Finally, the cells were supplied with 1 ml of fresh BME containing 1% fetal bovine serum and no antibiotics.

Interferon treatment of cell cultures and inoculation with bacteria. The cell cultures were treated with interferon for 24 h in concentrations from 10 to 1,000 U/ml. After 24 h the cells were washed with PBS and incubated for 1 h in fresh medium without interferon. The cultures were then inoculated with 200 μl of an *S. typhimurium* suspension that had been cultured overnight on heart infusion agar and suspended in PBS. The bacterial concentration was adjusted spectrophotometrically to ca. 2×10^7 bacteria per ml. Control cell cultures that were not treated with interferon were included. After incubation with bacteria, the cultures were washed in PBS and fixed overnight in 2% glutaraldehyde in 0.1 M cacodylate buffer with 0.1 M sucrose.

Microscopical examination. Bacterial invasiveness in cell cultures was quantified by counting intracellular bacteria in each of the parallel samples. To ensure that our results were reflecting invasiveness and not merely differences in bacterial adhesiveness, the cell cultures were examined in a combined-light microscopical system which allows differentiation between extracellular and intracellular localizations of bacteria. This system (4) combines Nomarski differential-interference contrast microscopy and UV incident-light microscopy applied on the same microscope. The UV incident-light microscopy gives the total number of bacteria in the cell culture, whereas the differential-interference contrast microscopy distinguishes between the different locations of the bacteria. The accuracy of the method for selecting extracellular bacteria has been confirmed by means of scanning electron microscopy in a previous study (3). In each of the two parallel cultures, 200 cells were examined for intracellular bacteria at a total magnification of ×1,000. The number of bacteria per cell was recorded. Statistical differences were evaluated by the chi-square test.

Bacteriostatic effect of interferon. Tubes with 2 ml of BME with and without 20% mouse serum and tubes with BME and 500 U of interferon per ml, with and without 20% mouse serum, were incubated with 250 μl of *S. typhimurium* (2.3×10^6 bacteria per ml) at 37°C for 3 and 24 h. After incubation, 10-fold dilutions of the samples were inoculated on lactose agar plates, and the number of bacteria was recorded.

RESULTS

Interferon effect on mortality of mice challenged with *S. typhimurium*. Mice that had been given *S. typhimurium* intragastrically became sick and died 12 to 18 h after the administration. The dose of live bacteria necessary to kill 75% of the mice varied from 5.0×10^7 to 3.0×10^8 bacteria per mouse from one experiment to another. This variation made it necessary to use several concentrations of bacteria in each experiment to be sure to obtain at least one control group presenting between 50 and 80% mortality. When the mortality rate was kept within this range in the control group, the mortality rate in the interferon-treated group was significantly reduced. A representative experiment is shown in Table 1. Interferon and bacteria were given concomitantly, the infectious dose being 1.3×10^8 live bacteria per ml. In the control group 13 out of 17 mice died (66%), compared with 2 out of 17 (12%) in the interferon-treated group 22 h after inoculation with bacteria. In another experiment the infectious dose was raised to 5×10^8 bacteria per ml, and groups were treated with 100 and 1,000 U of interferon (Table 2). Treatment with 100 U of interferon per animal resulted in a delay in the development of disease compared with controls. After 16 h, 7 out of 10 mice were alive in the interferon group, whereas in the control group only 1 mouse had survived. After 24 h, however, all the mice were dead in the group treated with 100 U of interferon. When the animals were given 1,000 U of interferon, 6 out of 10 mice survived the bacterial challenge. When the mice were treated with an interferon preparation (0.2 ml of 5,000 U/ml per animal, i.e., 1,000 U per animal) that had been neutralized with anti-interferon globulin, we could not record any reduction in lethality. Out of 20 mice given neutralized interferon preparation, 16 mice survived, whereas 14 out of 20 mice survived in the control group. In the same experiment all 20 mice survived in the interferon-treated group (0.2 ml of 5,000 U/ml per animal).

Mortality of mice after inoculation with heat-inactivated and freeze-pressed suspensions of *S. typhimurium*. To demonstrate the importance of viable bacteria for the development of disease, the mortality rate for mice given heat-inactivated bacteria and freeze-pressed bacteria was compared with that of mice given crude bacterial suspensions. All 10 mice that were given heat-inactivated bacteria survived, whereas all 10 mice that were given live bacteria died after 24 h. The freeze-

TABLE 1. Effect of mouse fibroblast interferon on mortality of infant mice after challenge with *S. typhimurium*[a]

Interferon dose (U/animal)	Time after bacterial inoculation (no. of live mice/total no. of mice)		
	12 h	18 h	22 h
0	13/17	7/17	4/17
500	16/17	16/17	15/17

[a] $P < 0.001$, chi-square test.

press treatment reduced the number of infectious particles from 2.9×10^8 per ml in the crude preparation to 6.5×10^5 per ml in the freeze-pressed preparation. This corresponds to a reduction of the number of infectious particles to 0.22% of the original dose. The mortality rate for mice given the freeze-pressed preparation was significantly reduced compared with the mortality rate for mice given the original preparation. In the group that received freeze-pressed material, 9 out of 45 mice died, whereas in the control group 35 out of 45 mice died (Table 3). In the groups given the two highest concentrations of bacteria, 28 out of 30 mice died in the control group compared with 8 out of 30 in the group given freeze-pressed bacteria.

Isolation of bacteria from the liver after inoculation with *S. typhimurium*. To examine whether the difference in mortality rates between the interferon groups and the control groups reflected a delayed spread of bacteria into the blood stream, samples from the liver parenchyme were examined for bacterial growth at different times after inoculation. The animals were treated with 1,000 U of interferon. High (1.8×10^8 bacteria per ml) and low (1.3×10^5 bacteria per ml) concentrations of bacteria were inoculated. Samples taken from the liver after 20 min, 40 min, 1 h, 2 h, 3 h, and 4 h showed no difference between the interferon-treated and the control groups. As early as 20 min after bacterial inoculation, bacteria could be grown from the tissue samples in both groups.

In vivo invasiveness of *S. typhimurium* in the small intestine epithelial cells of mice. No bacteria could be observed intracellularly in epithelial cells in the smear from the small intestine of the mice 30 min after intragastrical administration. All five mice in the control group had intracellularly infected epithelial cells 90 min after administration of bacteria. From 5 to 22% of the counted cells harbored intracellular bacteria (mean, 15.6%). In the interferon-treated animals, intracellular bacteria could be observed in two out of five animals. Only 3 and 2%, respectively, of the cells harbored intracellular bacteria, giving a mean score of 1% in five animals. The results are summarized in Table 4.

In vitro invasiveness of *S. typhimurium* in mouse L-929 cells. The effect of interferon on invasiveness of cells was also tested in an in vitro system with monolayers of L-929 cells. After 5 h of incubation, ca. 20% of the cells were invaded by bacteria. The interferon-treated cultures harbored less intracellular *S. typhimurium* than the control cultures when interferon was added concomitantly with or before the bacteria. The combined results of two representative experiments are shown in Fig. 1. The interferon effect was dose dependent, although even 1 U of interferon significantly reduced the invasiveness. When antiviral activity of interferon was neutralized with specific antibodies, the inhibiting effect of the interferon preparation on in vitro invasiveness was also eliminated.

In vitro effect of interferon on growth of *S. typhimurium*. The effect of our interferon preparation on in vitro growth of *S. typhimurium* was tested. Tubes with BME, BME with 20% mouse serum, BME with 500 U of interferon per ml, and BME with 20% mouse serum and 500 U of interferon per ml all contained the same number of bacteria after 3 and 24 h of incubation.

TABLE 2. Effect of mouse fibroblast interferon on mortality of mice after challenge with *S. typhimurium*

Interferon dose (U/animal)	Time after bacterial inoculation (no. of live mice/total no. of mice)		
	16 h	20 h	24 h
0	1/10	1/10	1/10
100	7/10	2/10	0/10
1,000	6/10	6/10	6/10

TABLE 3. Mortality of mice after inoculation with freeze-pressed suspensions of *S. typhimurium*

Time after bacterial challenge (h)	No. of live mice[a] and dilution of bacterial suspension:					
	Before freeze pressing[b]			After freeze pressing[c]		
	1:1	1:2	1:4	1:1	1:2	1:4
2	14	15	15	15	14	15
4	14	15	15	15	13	15
6	14	15	14	15	13	15
8	10	13	12	15	12	15
16	1	1	8	11	11	13
24	1	1	8	11	11	12

[a] Out of groups of 15.
[b] A bacterial suspension containing 2.9×10^8 live bacteria per ml.
[c] A bacterial suspension containing 6.5×10^5 bacteria per ml.

DISCUSSION

The present data indicate that treatment of infant mice with homologous fibroblast interferon enhances the resistance to infection with *S. typhimurium*. The effect of interferon is dose dependent and can be inhibited by anti-interferon globulin. When the animals were given interferon doses of ca. 100 to 200 U/g, the mortality rate was reduced. When lower interferon doses were given, a delay in progress of disease could be observed.

The mechanisms for pathogenicity of *S. typhimurium* are not fully understood. In the early phase of infection *Salmonella* bacteria seem to infect the ileal mucosa and the Peyer's patches. With time this infection progresses to the draining lymph nodes and reaches the liver and the spleen (6). It is assumed that one of the major pathogenetic factors for development of disease is the ability of the bacteria to invade cells and survive and multiply intracellularly in the host organism. The presence of a possible exotoxin has been discussed in some experiments (20), but the importance of such toxins for pathogenicity is not clear. However, to exclude the possibility that the mortality of the mice observed in our experiment was due to liberation of endo- or exotoxigenic compounds, the animals were challenged with heat-inactivated and freeze-pressed bacterial suspensions. Mice challenged with heat-inactivated bacteria survived. We could not, however, rule out the possibility that the denatur-

TABLE 4. Effect of mouse fibroblast interferon on invasiveness of *S. typhimurium* in mouse small intestine epithelial cells 90 min after intragastrical administration of the bacteria

Animal no.	Interferon dose (U/animal)	Infected cells (%)
1	0	5
2	0	17
3	0	15
4	0	17
5	0	22
6	1,000	0
7	1,000	0
8	1,000	0
9	1,000	2
10	1,000	3

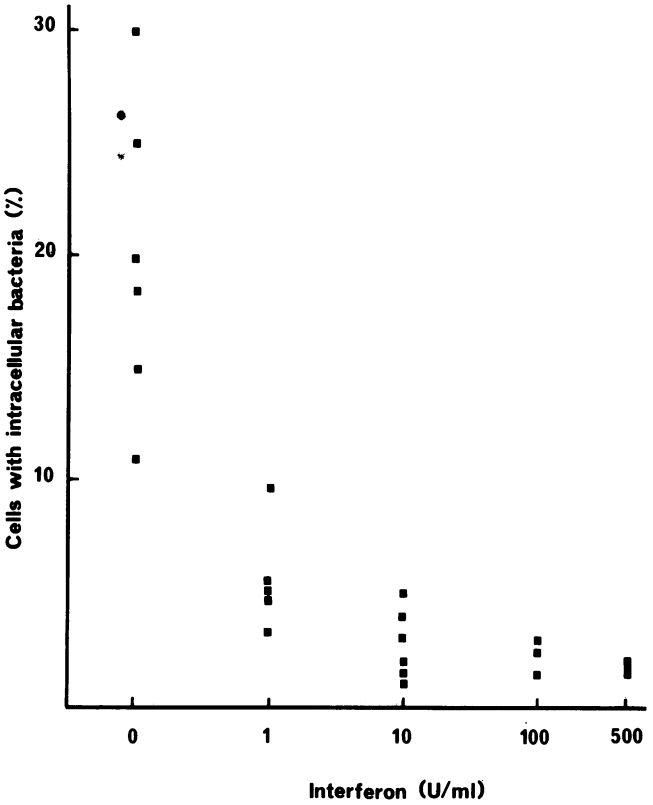

FIG. 1. In vitro invasiveness of *S. typhimurium* in mouse L-929 cells after pretreatment of the cells with various concentrations of mouse fibroblast interferon. Each point represents a count of 200 cells. The ratio of cells containing intracellular bacteria decreased when the interferon titer was raised from 1 U/ml to 500 U/ml.

ation of proteins that follows heat inactivation also included toxigenic compounds. Therefore the experiment was repeated with freeze-pressed bacteria, a treatment which denaturates bacterial proteins to a much lesser extent. The number of live bacteria was reduced to 0.22% after freeze pressing compared with the crude suspension, whereas the mouse mortality rate sank from 93 to 27%. We were not able to kill 100% of the bacteria by freeze pressing, and this probably explains why 3 to 4 out of 15 mice died after the treatment. However, the difference in mortality between the two groups can primarily be explained by differences in the infectious dose, since the mass of bacterial substance given to the two groups of mice was equal.

In a previous study we have shown that invasiveness of *S. typhimurium* in HEp-2 cell cultures (derived from human tissue) could be inhibited by interferon (3). In the present study this in vitro effect has been further confirmed to be valid in a mouse in vitro system. The bacterial invasiveness in mouse L-929 cells was significantly reduced when cell cultures were treated with interferon. The effect was dose dependent and specific.

Several studies have shown that there is a correlation between invasiveness in vitro and in vivo pathogenicity (5, 11) for *S. typhimurium*. It is generally accepted that the ability to internalize into cells and multiply there is one of the most important pathogenetic factors for bacteria causing human or mouse typhoid fever. Therefore, it is reasonable to correlate the interferon effect shown in the in vitro mouse L-929 system with the in vivo effect, i.e., the reduction of lethality to mice. In addition we showed that interferon inhibits the invasiveness of *S. typhimurium* in small intestinal epithelial cells in mice in vivo. It is reasonable to assume that this reduced invasiveness in vivo accounts for the reduced lethality. However, we found no significant difference between the interferon-treated group and the control group when investigating how soon after infection we could isolate bacteria from the liver. We cannot exclude the possibility of an early spread of bacteria from esophagus to the liver, although the intubation was performed very gently and the animals were always allowed to swallow the tube. When mice were inoculated with the same volume of a tryphan blue-stained NaCl solution, the stain was located in the small intestine immediately after injection. Izadkhah et al. (14) have earlier shown that type II interferon reduces the mortality of mice given *S. typhimurium* intraperitoneally and that bacteria could be frequently isolated from different tissues regardless of interferon treatment. In that experiment the spread of bacteria did not seem to be inhibited by interferon. It is clear that interferon inhibits the uptake of bacteria in intestinal cells in vivo and in mesenchymal-derived cells (L-929) in vitro. This may indicate that the virulence of the infection is reduced by inhibited bacterial uptake in intestinal cells and delayed establishment of the intracellular state of infection.

However, interferon is also known to influence a number of other host defense mechanisms against bacterial infection. In vitro studies have shown that uptake and degradation of bacteria by macrophages are enhanced by interferon treatment (2, 9, 12, 19). We also know that the immune response will be modified after interferon treatment (23, 24). It has not been possible to quantify the significance of these factors in our in vivo experiment. The choice of infant mice as our in vivo model was to minimize these factors because of the immaturity of the infant mouse immune system. The ability of intracellular parasites to invade cells and survive intracellularly is an important virulence factor because an intracellular location protects them against the activity of extracellular antiinfection defense mechanisms. Any effect that inhibits this internalization makes the extracellular bacteria more exposed to macrophages, granulocytes, and immune factors.

In conclusion, interferon is shown to inhibit the invasiveness of *S. typhimurium* in vitro as well as in vivo in mouse cell systems. The inhibition of invasiveness might be a major mechanism when interferon increases the resistance of mice to *S. typhimurium* infection.

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