

## Effect of Human Leukocyte Interferon on Invasiveness of *Salmonella* Species in HEp-2 Cell Cultures

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The effect of human leukocyte interferon on the invasiveness of *Salmonella* and *Shigella* species in HEp-2 cell cultures was examined. The intracellular and extracellular bacteria were identified by a combination of Nomarski differential interference contrast microscopy and UV incident light microscopy applied on the same microscope. Pretreatment of HEp-2 cells with human leukocyte interferon reduced the number of *Salmonella typhimurium* and *Salmonella paratyphi-B* bacteria per cell and the proportion of cells containing bacteria in a dose-dependent manner. Maximum inhibitory effect was observed with ca. 100 U of interferon per ml. The inhibitory effect was neutralized with anti-human interferon globulin. Murine fibroblast interferon did not influence the invasiveness of *Salmonella* species. Invasiveness of *Shigella flexneri* was not influenced by treatment of cells with human interferon.

Bacterial invasiveness in cell culture monolayers has been found to reflect an important pathogenic property for the enteroinvasive *Shigella* bacteria (9, 12), invasive *Escherichia coli* (25), *Salmonella* (13), and *Yersinia enterocolitica* (32). The ability to invade HEp-2 and HeLa cells is apparently well correlated with the potential to produce disease in humans for *Shigella*, *Salmonella typhimurium*, and invasive *E. coli* strains. For human pathogenic *Y. enterocolitica* serotypes O:3 and O:9, the correlation seems less complete; thus, it is reported that removal of a plasmid connected with human disease does not reduce the in vitro invasiveness of the bacteria (33). The cell monolayer model thus reflects a restricted number of factors involved in the pathogenic mechanisms of these bacteria. This model is suitable for examining the influence of extracellular factors on bacterial invasiveness.

Interferon is frequently present in infections with bacteria and other infectious agents (1, 7, 19, 27, 29). It has been shown that interferon can influence several host defense mechanisms against bacterial infections, e.g., the uptake of and degradation of bacteria by macrophages and granulocytes (2, 8, 14), and modify immune response to various infectious agents (30). Other studies have indicated that mouse fibroblast interferon can modify the pulmonary infection of mice infected with *Haemophilus influenzae* (8) and that immune interferon may reduce the mortality due to *S. typhimurium* in mice (18). In this study, we examined the direct effect of interferon on the ability of epithelial cell barriers to restrict infections with invasive bacteria. Pretreatment of cells with human leukocyte inter-

feron reduced the invasiveness of *S. typhimurium* in HEp-2 cells in a dose-dependent manner.

Monolayers of HEp-2 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.  $6 \times 10^4$  cells in Eagle basal medium containing bicarbonate, 10% fetal bovine serum, and 100  $\mu$ g of streptomycin and 100  $\mu$ g of penicillin per ml. They were incubated for approximately 24 h at 37°C in 5% CO<sub>2</sub> 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 Eagle basal medium, and then washed three times again with PBS to minimize the concentration of antibiotics. Finally, the cells were supplied with 1 ml of fresh Eagle basal medium containing 1% fetal bovine serum and no antibiotics.

One *S. typhimurium* strain (SIF 54575/81), one *Salmonella paratyphi-B* strain (strain 3, 8006; International Salmonella Center, Copenhagen) and one *Shigella flexneri* strain (SIF 662/81) were used. The strains of *S. typhimurium* and *S. flexneri* were invasive in HEp-2 cell monolayer, and the *S. flexneri* strain was able to produce keratitis in guinea pigs (Serény test) (5).

Partially purified human leukocyte interferon ( $\alpha + \beta$ ;  $2 \times 10^6$  U per mg of protein) was obtained from K. Cantell, Helsinki. The antiviral activity was tested by means of an infectivity inhibition microtest (6) with human embryo fi-

broblast cells and vesicular stomatitis virus. One unit of the international standard preparation (69/19) was equivalent to 1.0 to 1.3 U in our system. Anti-human interferon globulin was obtained from J. K. Dunnick, National Institute of Allergy and Infectious Diseases, Bethesda, Md. A 1:1,000 dilution neutralized 10 U of interferon. Mouse fibroblast interferon ( $\alpha + \beta$ ) was obtained from the late K. Paucker, Philadelphia, Pa.

The cell cultures were treated with interferon for 24 h in concentrations from 50 to 10,000 U per ml. After 24 h they were washed with PBS and incubated for 1 h in fresh medium without interferon. The cultures were then inoculated with 200  $\mu$ l of a bacterial suspension that had been cultured on heart infusion agar and suspended in PBS. The concentration was adjusted to an optical density of 0.75 at 320 nm (Hitachi model 101 spectrophotometer). Control cultures not treated with interferon were included for each bacterial strain. After incubation for 3 h with the bacteria, the cultures were washed in PBS and fixed overnight in 2% glutaraldehyde in 0.1 M cacodylate buffer with 0.1 M sucrose. The *S. flexneri* culture was incubated for another 2 h before fixation.

Bacterial invasiveness was quantified by counting intracellular bacteria in each of the parallel samples. To ensure that our results reflected invasiveness and not merely differences in bacterial adhesiveness, we examined the cell cultures 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 (4). In each of two parallel cultures, 200 cells were examined for intracellular bacteria at a total magnification of  $\times 1,000$ . The microscope slides were coded before reading in the microscope, so the microscopist did not know which treatment had been given to the specimen. The number of bacteria per cell was recorded. Statistical differences were evaluated by the chi-square test.

After 3 h of incubation, a considerable number of cells were invaded by bacteria. The interferon-treated cultures harbored less intracellular *S. typhimurium* than the control cultures. This effect was dose dependent. The distribution of *S. typhimurium* in the cells at various concentra-

tions of interferon is shown in Fig. 1. Treatment with low concentrations of interferon entailed a shift of the distribution to the left in Fig. 1. Even the lowest concentration of interferon, 50 U/ml, gave a statistically significant shift. The maximum inhibitory effect was observed with 100 U of interferon per ml. At two consecutive concentrations, 500 and 1,000 U/ml, a shift toward the right on the X-axis was observed compared to the effect of 100 U, and thus the inhibitory effect was less complete. With 10,000 U, an even further shift to the right was seen, and this high dosage gave the lowest inhibitory effect. When bacterial invasiveness is characterized by single parameters, the degree of inhibitory effect can be further illustrated (Table 1). The maximum inhibitory concentration of interferon (100 U/ml) reduced the number of intracellular *S. typhimurium* per 100 cells from 160 to 6 and the proportion of cells infected from 32 to 2.4%.

To test the specificity of the anti-invasive activity of interferon, we pretreated cell cultures with 100 U of human interferon per ml that had been neutralized with anti-human interferon globulin. The neutralized interferon had no significant effect on invasiveness of *S. typhimurium* (Table 1). To test for species specificity of interferon, we also treated the cultures with heterologous mouse fibroblast interferon at a concentration of 100 U/ml. This treatment also did not significantly alter the invasiveness pattern of *S. typhimurium* compared to control cultures.

To examine whether the effect on invasiveness of *S. typhimurium* was specific for that species of the *Salmonella* genus exclusively, we tested *S. paratyphi-B* for invasiveness in cell cultures pretreated with concentrations of interferon that gave the maximum inhibition of *S. typhimurium*. When the cell monolayer was preincubated with interferon at concentrations of 100, 200, and 300 U/ml, *S. paratyphi-B* was significantly inhibited in penetrating the cell cultures (Table 1).

The effect of interferon on the invasiveness of *S. flexneri* was tested to elucidate whether the inhibitory effect was restricted to bacteria within the genus *Salmonella* or whether this phenomenon was a more common characteristic of enteroinvasive bacteria. When cell cultures were pretreated with 500 U of human interferon per ml, no significant effect on the invasiveness of *S. flexneri* was observed (Table 1).

The present data clearly show that treatment of HEp-2 cells with homologous leukocyte interferon significantly reduces the invasiveness of the *Salmonella* species tested. Both the proportion of cells infected and the number of intracellular bacteria per cell were decreased in the interferon-treated cells. The biphasic effect of

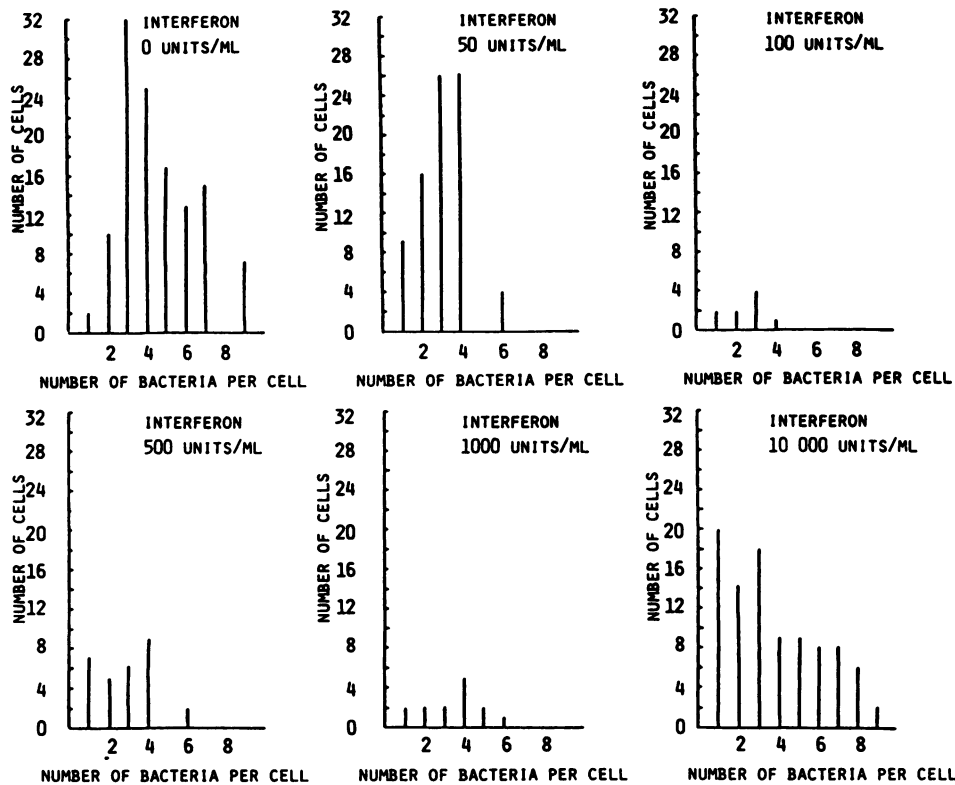


FIG. 1. Number of intracellular *S. typhimurium* bacteria in HEp-2 cells after pretreatment of HEp-2 cells with various concentrations of interferon.

interferon is very similar to its other biological effects, e.g., on phagocytosis and antibody response (2, 28, 31). The number of observed intracellular bacteria is the sum of several processes, namely, adhesion and penetration of bacteria and to some extent intracellular multiplication. Any of those parameters might be influenced by interferon.

Adherence of bacteria is mediated by specific receptors on the cell surface (20, 21, 24). Several receptor structures are influenced by interferon treatment. The expression of some receptors is apparently stimulated specifically, whereas other receptors are not (22, 23). To our knowledge there are no published data indicating a suppression of receptors by interferon. The observation that the invasiveness of *Shigella* species is not inhibited by interferon treatment like that of *Salmonella* is easiest to explain by a possible different effect on receptors for these bacteria. Further studies on this question are in progress in our laboratory.

The mechanisms by which the bacteria penetrate the cell membrane are not clearly understood. Both bacterial and host cell factors are of importance (16, 17). Our data clearly point to the importance of cellular mechanisms. Several ear-

TABLE 1. Invasiveness of *S. typhimurium*, *S. paratyphi-B*, and *S. flexneri* in HEp-2 cells treated with interferon

Species	Interferon <sup>a</sup> concn (U/ml)	% Infected cells	No. of bacteria per infected cell	No. of bacteria per 100 cells
<i>S. typhimurium</i>	0	32	5.0	160
	50	34	4.3	110
	100	2.4	2.3	6
	500	7.0	2.9	22
	1,000	6.0	5.9	36
	10,000	19	3.5	68
	100 <sup>b</sup>	33	5.2	173
	100 <sup>c</sup>	27	5.3	144
<i>S. paratyphi-B</i>	0	34	5.4	185
	100	7	3.5	25
	200	2	7.5	15
	300	3	5.7	17
<i>S. flexneri</i>	0	30	10.7	320
	500	29	9.8	284

<sup>a</sup> Human leukocyte interferon, except where indicated.

<sup>b</sup> Neutralized with anti-interferon globulin.

<sup>c</sup> Murine fibroblast interferon.

lier reports indicate that interferon alters the functional properties of the cell membrane. Thus, interferon impaired cell motility, increased plasma membrane rigidity, and reduced lateral movement of some cell surface receptors (26). After interferon treatment, the cell membrane seems to be less permeable to some low-molecular-weight substances (3, 11, 26), and retrovirus budding appears to be inhibited (10). The reduced penetration of invasive bacteria may be due to the same phenomenon. Whether these membrane changes are primary effects or secondary to an intracellular alteration of a metabolic process remains to be determined.

The possibility of an effect on the intracellular multiplication of bacteria cannot be excluded. In fact, interferon treatment inhibited the intracellular multiplication of several nonviral agents, including bacteria (15). However, the finding that not only the total number of bacteria per cell but also the percentage of cells containing bacteria is reduced, argues against this alternative.

The present observations may indicate that, in addition to activation of specific host defense mechanisms, interferon may influence the entry of bacteria into cells and thereby directly interfere with the establishment of bacterial infection.

#### LITERATURE CITED

- Baron, S., V. Howie, M. Langford, E. M. MacDonald, G. J. Stanton, J. Reitmeyer, and D. A. Weigent. 1982. Induction of interferon by bacteria, protozoa and viruses: defensive role. *Tex. Rep. Biol. Med.* 41:150-157.
- Braun, W., and M. B. Levy. 1972. Interferon preparations as modifiers of immune response. *Proc. Soc. Exp. Biol. Med.* 141:769-773.
- Brouty-Boyé, D., and B. R. Zetter. 1980. Inhibition of cell motility by interferon. *Science* 208:516-518.
- Bukholm, G., B. V. Johansen, E. Namork, and J. Lassen. 1982. Bacterial adhesiveness and invasiveness in cell culture monolayer. 1. A new light optical method evaluated by scanning electron microscopy. *Acta Pathol. Microbiol. Immunol. Scand. Sect. B* 90:403-408.
- Bukholm, G., and J. Lassen. 1982. Bacterial adhesiveness and invasiveness in cell culture monolayer. 2. Invasiveness of 45 strains belonging to the family *Enterobacteriaceae*. *Acta Pathol. Microbiol. Immunol. Scand. Sect. B* 90:409-413.
- Dahl, H., and M. Degré. 1972. A micro assay for mouse and human interferon. *Acta Pathol. Microbiol. Scand. Sect. B* 80:863-870.
- Degré, M. 1972. Effect of a standard bacterial vaccine on infection with an obligate intracellular parasite (*Toxoplasma gondii*) in mice. *Acta Pathol. Microbiol. Scand. Sect. B* 80:478-480.
- Degré, M., and H. Rollag, Jr. 1980. Pathogenesis of Sendai virus infection in mice. *Acta Pathol. Microbiol. Scand. Sect. B* 88:177-181.
- Formal, S. B., H. L. DuPont, R. B. Hornick, M. J. Snyder, J. Libonati, and E. H. LaBrec. 1971. Experimental models in the investigation of the virulence of dysentery bacilli and *Escherichia coli*. *Ann. N.Y. Acad. Sci.* 176:190-196.
- Friedmann, R. M. 1977. Antiviral activity of interferons. *Bacteriol. Rev.* 41:543-567.
- Friedmann, R. M. 1982. Effects of interferons on cell membranes. *Tex. Rep. Biol. Med.* 41:313-316.
- Gerber, D. F., and H. M. S. Watkins. 1961. Growth of shigellae in monolayer tissue cultures. *J. Bacteriol.* 82:815-822.
- Gianella, R. A., O. Washington, P. Gemski, and S. B. Formal. 1973. Invasion of HeLa cells by *Salmonella typhimurium*: a model for study of invasiveness of *Salmonella*. *J. Infect. Dis.* 128:69-75.
- Giron, D. J., J. P. Schmidt, R. J. Ball, and F. F. Pindak. 1972. Effects of interferon inducers and interferon on bacterial infections. *Antimicrob. Agents Chemother.* 1:80-81.
- Gober, L. L., A. E. Friedmen-Kien, E. A. Havell, and J. Vilcek. 1972. Suppression of the intracellular growth of *Shigella flexneri* in cell cultures by interferon preparations and polyinosinic-polycytidylic acid. *Infect. Immun.* 5:370-376.
- Hale, T. L., and P. F. Bonventre. 1979. *Shigella* infection of Henle intestinal epithelial cells: role of the bacterium. *Infect. Immun.* 24:879-886.
- Hale, T. L., E. Morris, and P. F. Bonventre. 1979. *Shigella* infection of Henle intestinal epithelial cell: role of the host cell. *Infect. Immun.* 24:887-894.
- Izadkhan, Z., A. D. Mandel, and G. Sonnenfeld. 1980. Effects of treatment of mice with sera containing gamma interferon on the course of infection with *Salmonella typhimurium*. *J. Interferon Res.* 1:137-145.
- Jahiel, R. I., J. Vilcek, R. Nussenzweig, and J. Vanderberg. 1968. Interferon inducers protect mice against *Plasmodium berghei* malaria. *Science* 161:802-804.
- Källenius, G., S. B. Svenson, R. Møllby, T. Korhonen, J. Winberg, B. Cedergren, I. Helin, and H. Hultberg. 1982. Carbohydrate receptor structure recognized by uropathogenic *E. coli*. *Scand. J. Infect. Dis. Suppl.* 33:52-60.
- Leffler, H., H. Lomberg, E. Gotschlich, L. Hagberg, U. Jodal, T. Korhonen, B. E. Samuelson, G. Schoolnik, and C. Svanborg-Eden. 1982. Chemical and clinical studies on the interaction of *Escherichia coli* with host glycolipid receptors in urinary tract infection. *Scand. J. Infect. Dis. Suppl.* 33:46-51.
- Lindahl, P., P. Leary, and I. Gresser. 1972. Enhancement by interferon of the specific cytotoxicity of sensitized lymphocytes. *Proc. Soc. Natl. Acad. Sci. U.S.A.* 69:721-725.
- Lindahl, P., P. Leary, and I. Gresser. 1973. Enhancement by interferon of the expression of surface antigens on murine leukemia L 1210 cells. *Proc. Soc. Natl. Acad. Sci. U.S.A.* 70:2785-2788.
- Lomberg, H., P. Larsson, H. Leffler, and C. Svanborg-Eden. 1982. Different binding specificities of *P. mirabilis* compared to *E. coli*. *Scand. J. Infect. Dis. Suppl.* 33:37-42.
- Mehlman, I. J., E. L. Eide, A. C. Sanders, M. Fishbein, and C. G. Aulisio. 1977. Methodology for recognition of invasive potential of *Escherichia coli*. *J. Assoc. Off. Anal. Chem.* 60:546-562.
- Pfeffer, L. M., E. Wang, and I. Tamm. 1980. Interferon effect on microfilament organisation, cellular fibronectin distribution and cell motility in human fibroblasts. *J. Cell. Biol.* 85:9-17.
- Remington, J. S., and T. C. Merigan. 1968. Interferon: protection of cells infected with an intracellular protozoan (*Toxoplasma gondii*). *Science* 161:804-806.
- Rollag, H., and M. Degré. 1981. Effect of interferon on the uptake of non-opsonized *Escherichia coli* by mouse peritoneal macrophages. *Acta Pathol. Microbiol. Scand. Sect. B* 89:153-159.
- Schultz, R. M., K. Y. Huang, and F. B. Gordon. 1968. Role of interferon in experimental mouse malaria. *Nature (London)* 220:709-710.
- Sonnenfeld, G., and T. C. Merigan. 1979. A regulatory role for interferon in immunity. *Ann. N.Y. Acad. Sci.*

- 332:345-355.
31. Stannegård, Ø., I. Larsson, E. Lundgren, H. Miørner, and H. Persson. 1977. Modulation of immune responses in newborn and adult mice by interferon. *Infect. Immun.* **20**:334-339.
32. Une, T. 1977. Studies on the pathogenicity of *Yersinia enterocolitica*. II. Interaction with cultured cells *in vitro*. *Microbiol. Immunol.* **21**:365-377.
33. Vesikari, T., T. Nurmi, M. Maki, M. Skurnik, C. Sundquist, and P. Grønroos. 1981. Plasmids in *Yersinia enterocolitica* serotypes O:3 and O:9: Correlation with epithelial cell adherence *in vitro*. *Infect. Immun.* **33**:870-876.