

## Natural and Recombinant Interferons Inhibit Epithelial Cell Invasion by *Shigella* spp.

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**The effect of natural and recombinant interferons (IFNs) on the abilities of *Shigella flexneri*, *S. sonnei*, and *Salmonella typhimurium* to invade different human and murine cells was examined. Pretreatment of cell monolayers with natural and recombinant IFNs reduced the number of *Shigella*-infected cells in a dose-dependent manner. Establishment of an anti-invasive cellular state was time dependent, requiring 10 h for 50% inhibition of bacterial invasion. The inhibitory effect of IFN was species specific, with human or murine IFN effective against homologous but not heterologous cells. Gamma IFN was slightly more potent than alpha IFN at inhibiting bacterial invasion. Inhibition of *Shigella* invasion was dependent on the challenge dose of bacteria. Little inhibition of invasion was seen when cells were pretreated with low concentrations of IFN and challenged with high multiplicities of infection of *Shigella* sp. In contrast to *Shigella* invasion, the maximum inhibitory effect of IFN on *Salmonella* invasion of cells was observed at low levels (5 to 50 U) of IFN. These results suggest that *Shigella* and *Salmonella* invasions occur at unique sites on eucaryotic cells or by different penetration mechanisms. More importantly, these data suggest that IFN may play a significant role in host defense against *Shigella* and *Salmonella* infections.**

The role of interferon (IFN) in host defense against viral infections has been well established. In addition, evidence has accumulated which suggests that IFNs may play an important role in host defense against bacterial infections. Numerous investigators have demonstrated that bacteria, bacterial products, and in some instances both can induce IFN- $\gamma$  or IFN- $\alpha$  from different cells of the immune system (15, 21, 22, 37, 38, 40, 41). IFN- $\gamma$  plays an important role in macrophage activation (20, 23). The bacteriostatic and bacteriocidal activities of these activated macrophages are believed to be a major mechanism of host defense against bacterial infections (19, 29). Additionally, we (17a) have recently demonstrated that peripheral blood mononuclear cells can mediate natural cytotoxic activity against *Shigella*-infected cells and that this cytotoxic activity can be enhanced by IFN- $\alpha$ . Recently, a new role for IFN in host defense against facultative intracellular bacterial infections has been described. Pretreatment of different epithelial cell lines with either IFN- $\alpha$  or IFN- $\gamma$  has been shown to inhibit invasion by different *Salmonella* spp. (7, 8). However, it is still unclear as to whether IFN can inhibit the invasion or intracellular replication of other facultative intracellular bacteria. Gober et al. (14) showed that pretreatment with poly(I) · poly(C) but not IFN significantly reduced *Shigella flexneri* invasion in human foreskin cells. However, IFN and poly(I) · poly(C) were shown to reduce intracellular *Shigella* replication significantly. Recently, Bukholm and Degré (7, 8) have reported that pretreatment of HEp-2 cell monolayers with IFN- $\alpha$  or IFN- $\gamma$  did not inhibit the invasion of *S. flexneri* or *Yersinia enterocolitica* as determined by microscopic examination of pretreated monolayers.

The question of whether IFN can inhibit cellular invasion by bacteria is extremely important, since the virulence of many facultative intracellular bacteria is dependent on the ability of these organisms to penetrate and replicate within epithelial cells. The abilities of *Salmonella* spp. (12, 13),

*Shigella* spp. (18, 26, 28), invasive *Escherichia coli* (10, 26, 27), and to a lesser extent *Yersinia enterocolitica* (39) to invade epithelial cells correlate highly with their abilities to produce disease in humans. For *Shigella* (35, 36) and *Salmonella* spp. (12, 13), those strains which lose the ability to penetrate epithelial cells are uniformly avirulent in animal models.

Recently, we have developed an assay for the detection and quantitation of bacteria-infected cells (24). This method uses antibiotic counterselection to remove noninternalized bacteria and is significantly more sensitive than existing microscopic methods. Utilizing this assay, we show that natural and recombinant IFNs inhibit the penetration of epithelial cells by *S. flexneri* and *S. sonnei* in a dose- and time-dependent manner.

### MATERIALS AND METHODS

**Cell culture and media.** HeLa, HEp-2, and mouse L929 cells were maintained as monolayers in RPMI 1640 medium supplemented with 5% fetal calf serum, 2 mM glutamine, 50 U of penicillin per ml, and 50  $\mu$ g of streptomycin per ml. Mouse embryo fibroblasts (MEF) were prepared from 10-day-old embryos obtained from C57BL/6 mice (Jackson Laboratory, Bar Harbor, Maine). Briefly, minced tissue was trypsin digested for 15 min and then neutralized with fetal calf serum (M.A. Bioproducts, Walkersville, Md.). Cells ( $10^8$ ) were suspended in supplemented HMEM (10% fetal calf serum, 100 U of penicillin per ml, 100  $\mu$ g of streptomycin per ml) and seeded into 150-ml sterile tissue culture flasks (75 cm<sup>2</sup>). Cells were passaged weekly for up to 6 weeks. Cell monolayers were established in 24-well plates ( $2 \times 10^5$  per well) using the above cell lines.

**Bacterial strains.** An invasive *S. flexneri* serotype 2a strain (SA100) was used for challenging cell monolayers and has recently been described (31). *S. flexneri* 5348 and *S. sonnei* 7383 were obtained from G. Buck, Department of Pathology, University of Texas Medical Branch, Galveston. *Salmonella typhimurium* TML has been previously described (12). Bac-

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terial cultures were passaged on L or Congo red agar (30) and started from frozen stocks monthly. Prior to cell monolayer invasion, overnight bacterial cultures were split 1/100 into brain heart infusion broth (Difco Laboratories, Detroit, Mich.) and grown with aeration at 37°C to mid-logarithmic phase (optical density at 600 nm = 0.6).

For all experiments except where indicated, the number of infected cells in a monolayer was determined by our recently published agarose-agar overlaying procedure (24). Prior to invasion, mid-logarithmic bacterial cultures were washed and suspended in RPMI 1640 supplemented with 0.67 µg of FeCl<sub>3</sub> and 0.45% glucose. The level of bacterial invasion for these experiments was controlled by varying the multiplicity of infection (MOI) (0.1 to 200 invasive bacteria per cell). *S. flexneri* and *S. typhimurium* were left in contact with cell monolayers for 60 and 90 min, respectively. Noninternalized *S. flexneri* was counterselected with 50 µg of kanamycin per ml for 2 h, and extracellular *S. typhimurium* was counterselected with 50 µg of kanamycin and 20 µg of gentamicin per ml for 2 h. *Shigella*-infected cells were overlaid with 300 µl of 0.5% agarose in distilled water followed by 300 µl of 2× L broth which contained 0.5% agar (0.5% 2× L agar). For *S. typhimurium*, cells were first overlaid with 300 µl of 1.5% agarose in distilled water followed by 300 µl of 2× L broth containing 1.5% agar (1.5% 2× L agar). Bacterial colonies detected after 24 h of incubation at 37°C represented single or adjacently infected HeLa cells. The percent reduction in the number of bacterium-infected monolayer cells was determined as follows:  $1 - \frac{[(\text{mean CFU in IFN-treated wells})/(\text{mean CFU in control wells})] \times 100}{100}$ . Counts represent the average of at least duplicate wells.

**IFN preparations.** Human natural IFN-α (nIFN-α) ( $2.3 \times 10^5$  U/ml) was obtained from Lee Biomolecular, San Diego, Calif., and human nIFN-γ ( $2.5 \times 10^5$  U/ml) was from Cellular Products, Buffalo, N.Y. Recombinant IFN-α (rIFN-α) ( $2 \times 10^5$  U/ml) was obtained from Schering Corp., Bloomfield, N.J., and rIFN-γ ( $2 \times 10^6$  U/ml) was obtained from Biogen Corp. Monolayers of HeLa or HEp-2 cells were treated with IFN preparations for 16 h prior to invasion except where indicated. MEF and L-cell monolayers were treated for 20 h prior to invasion. Approximately 1 h prior to invasion, IFN was removed, and monolayers were washed twice with RPMI 1640 with no antibiotics.

Neutralization of IFN-γ was performed by incubating nIFN-γ with monospecific IFN-γ antiserum or nonimmune serum at 27°C for 1 h prior to its introduction onto cell monolayers. Antiserum raised to nIFN-γ was the generous gift of G. J. Stanton, Department of Microbiology, University of Texas Medical Branch, Galveston, and has been previously described (16). Each milliliter of antiserum was capable of neutralizing 10,000 U of IFN-γ as determined by the prevention of IFN-γ-induced inhibition of Sindbis virus cytopathic effects in human amnion WISH cells (3).

Preparations of murine IFN-α/β ( $10^5$  U/ml) were generously provided by Samuel Baron, Department of Microbiology, University of Texas Medical Branch, Galveston. IFN titers were determined by plaque reduction of vesicular stomatitis virus on L cells by the method of Campbell et al. (9). One unit of IFN is defined as the amount that reduces the number of plaques by 50% compared with the virus control.

## RESULTS

We have previously shown that agarose-agar overlays of bacteria-infected cell monolayers represent a sensitive method to quantitate the number of bacteria-infected cells in a monolayer (24). The number of bacteria-infected cells in a

TABLE 1. IFN inhibits invasion of HeLa cells by *Shigella* spp.

Bacterial strain	Pretreatment of HeLa cells <sup>a</sup>	Mean % reduction of infected HeLa cells <sup>b</sup>
Expt 1		
<i>S. flexneri</i> SA100	Human rIFN-γ (200 U)	52
<i>S. flexneri</i> 5348	Human rIFN-γ (200 U)	63
<i>S. sonnei</i> 7583	Human rIFN-γ (100 U)	36
Expt 2		
<i>S. flexneri</i> SA100	Human nIFN-γ (400 U)	79
	Human nIFN-γ (400 U)	21
	+ anti-IFN-γ Human nIFN-γ (400 U) + NRS <sup>c</sup>	86
Expt 3		
<i>S. flexneri</i> SA100	Human rIFN-γ (200 U)	83
	Mouse nIFN-γ (200 U)	12

<sup>a</sup> nIFN and rIFN were diluted in RPMI 1640 to produce the indicated concentrations per well. Pretreatment of HeLa cell monolayers was for 16 h. No cytotoxicity to the monolayers was observed. Antiserum sufficient to neutralize 400 U of IFN-γ was used.

<sup>b</sup> Represents the average of at least duplicate wells.

<sup>c</sup> Normal rabbit serum.

monolayer is adjusted by varying the MOI of invasive bacteria. Treatment of monolayers for 60 min with *Shigella* spp. or for 20 min with *S. typhimurium* provided optimal bacterial invasion. This assay system provided a convenient method for evaluating the effect of natural and recombinant IFNs on bacterial invasion of cell monolayers.

The ability of IFN-γ to inhibit the invasion of different *Shigella* spp. in HeLa cell monolayers is shown in Table 1. HeLa cells pretreated with either nIFN-γ or rIFN-γ were protected from *Shigella* invasion. This protection was observed with both *S. flexneri* and *S. sonnei*. Antiserum against nIFN-γ inhibited the protective effect against *Shigella* invasion. Mouse IFN-γ had no effect on HeLa cell susceptibility to *Shigella* invasion, which shows that the protective effect mediated by IFN is species specific. These data strongly suggest that IFN pretreatment of HeLa cells inhibits invasion by *S. flexneri* and *S. sonnei*.

Figure 1 shows the effect of rIFN-γ and rIFN-α on *Shigella* invasion of HeLa cell monolayers at various MOIs of *Shigella* sp. The level of inhibition of *Shigella* invasion mediated by both rIFN-γ and rIFN-α was dependent on the MOI of *Shigella* sp. used for invasion. Optimal inhibition of *Shigella* invasion was observed when low numbers of *Shigella* sp. were used for invasion along with high IFN concentrations for the pretreatment of the HeLa cells. In fact, at high MOIs, little or no inhibition of *Shigella* invasion was observed.

Establishment of the cellular anti-invasive state by IFN was examined by pretreating HeLa cells with rIFN-γ for various periods prior to *Shigella* challenge. The maximum level of inhibition of *Shigella* invasion was observed when HeLa cells were pretreated with rIFN-γ for at least 16 h prior to bacterial challenge (Fig. 2). After 4 h of pretreatment, the number of *Shigella*-infected HeLa cells was reduced by 26%, whereas at 16 h the number of *Shigella*-infected HeLa cells was reduced by 75%. Thus, the kinetics of the establishment of the antibacterial invasion state appear to be similar to the kinetics for IFN-induced antiviral states (1).

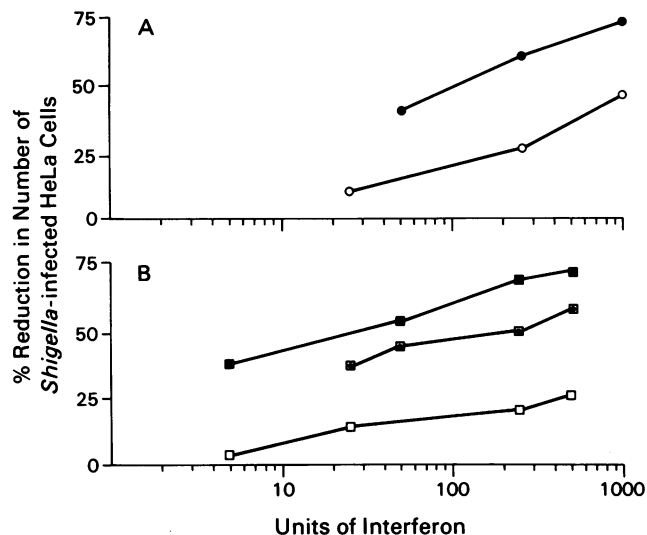


FIG. 1. Inhibitory effects of rIFN- $\gamma$  and rIFN- $\alpha$  on *Shigella* invasion at different challenge doses. (A) Duplicate wells were treated with 50, 250, or 1,000 U of rIFN- $\gamma$  for 16 h prior to *Shigella* challenge. The lower line (○) represents an MOI of 250:1 (bacteria: HeLa cells). There were 395 CFU per well in untreated control wells. The upper line (●) represents a challenge with an MOI of 25:1. Controls for this curve contained an average of 41 CFU per well. Variation in all counts was less than 15% of the average count. (B) Duplicate wells were treated with 25, 100, or 250 U of rIFN- $\alpha$  for 16 h prior to *Shigella* challenge. The lower line (□) represents an MOI of 1,000:1, with control wells containing an average of 1,230 CFU per well. The middle line (◻) represents an MOI of 100:1, with an average count in control wells of 88 CFU per well. The upper line (■) represents an MOI of 10:1, with an average count in control wells of 26.

IFN- $\gamma$  has been shown to have more potent antitumor, anticellular, and antiviral activities than IFN- $\alpha$  or IFN- $\beta$  (2). Therefore, we compared the relative effectiveness of nIFN- $\gamma$  versus nIFN- $\alpha$  on the inhibition of *Shigella* invasion of HeLa cells. IFN- $\gamma$  was shown to be slightly more effective than IFN- $\alpha$  in inhibiting *Shigella* invasion (Fig. 3).

The inhibitory effect of IFN on the ability of *Salmonella* sp. to invade HEP-2 cells has been previously reported (7, 8). To show that our assay system was applicable to other bacteria and other cell types, we examined the effect of rIFN- $\gamma$  pretreatment of HEP-2 and HeLa cells on *S. typhimurium* invasion. Pretreatment of HEP-2 or HeLa cells with rIFN- $\gamma$  inhibited *Salmonella* invasion (Table 2). Optimal inhibition of *Salmonella* invasion of HeLa or HEP-2 cells was observed at low IFN concentrations, whereas pretreatment of either cell type with high concentrations of IFN- $\gamma$  resulted in much less inhibition of *Salmonella* invasion. The data for HeLa cells are shown in Fig. 4. Essentially identical data were obtained for HEP-2 cells and are not shown. In contrast, *Shigella* invasion was inhibited in a dose-dependent fashion over the range of IFN concentrations tested.

The ability of IFN to inhibit *Shigella* invasion in nonhuman cells was also investigated by using MEF and mouse L-929 cells. MEF and L-cell monolayers were pretreated with murine IFN- $\alpha/\beta$  and challenged with *S. flexneri*. *Shigella* invasion of MEF and L cells was inhibited by pretreating these cells with either IFN- $\alpha/\beta$  or IFN- $\gamma$  (Table 3). Treatment of MEF monolayers with human rIFN- $\gamma$  did not inhibit *Shigella* invasion. These results clearly show that

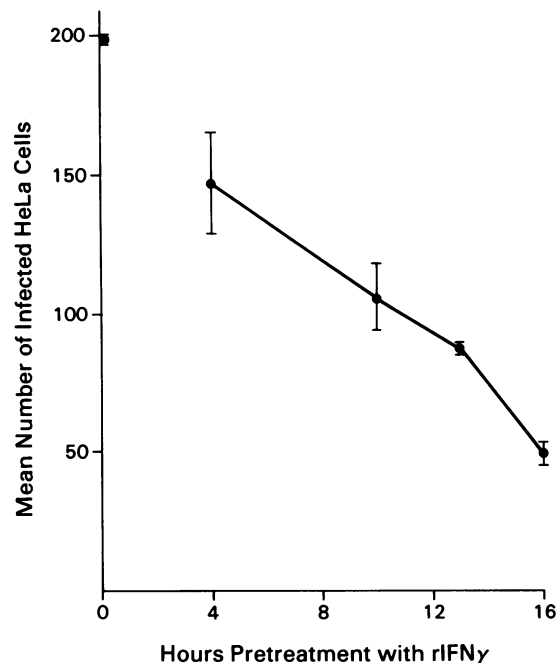


FIG. 2. Establishment of an anti-invasive state by rIFN- $\gamma$ . HeLa cell monolayers were treated with 500 U of rIFN- $\gamma$  at 4, 13, or 16 h before challenge with *S. flexneri* SA100 at an MOI of 100:1. *Shigella* sp. was allowed to invade for 60 min, and noninternalized bacteria were removed by counterselection with RPMI 1640 containing 50  $\mu$ g of kanamycin per ml for 2 h. Reported counts represent the average of two wells, and error bars show the standard deviation of the mean.

different cells pretreated with IFN become resistant to invasive pathogenic bacteria.

## DISCUSSION

The ability of *Salmonella* and *Shigella* strains to invade and replicate within different cell types has been demonstrated to be highly correlated with their *in vivo* invasiveness and their potential to cause disease in humans (13, 18). Previous studies have shown that the invasiveness of *Salmonella* spp. in cell monolayers can be inhibited by IFN pretreatment of the cells (7, 8). We showed in this study that

TABLE 2. Inhibition of *Salmonella* and *Shigella* invasion by rIFN- $\gamma$

Target cell	Bacterial strain	Pretreatment <sup>a</sup>	Infected cell count (CFU/well) <sup>b</sup>	% Reduction in infected HeLa cells
HEP-2	<i>S. typhimurium</i> TML	Medium	201 $\pm$ 10	45
		50 U of rIFN- $\gamma$	109 $\pm$ 8	
HeLa	<i>S. typhimurium</i> TML	Medium	119 $\pm$ 6	47
		10 U of rIFN- $\gamma$	62 $\pm$ 1	
HEP-2	<i>S. flexneri</i> SA100	Medium	395 $\pm$ 32	24
		250 U of rIFN- $\gamma$	300 $\pm$ 26	

<sup>a</sup> nIFN and rIFN were diluted in RPMI 1640 to produce the indicated concentrations per well. Pretreatment of HEP-2 and HeLa cell monolayers was for 16 h. IFN was removed, and monolayers were washed with antibiotic-free RPMI 1640 1 h before introduction of bacteria.

<sup>b</sup> Counts of infected HeLa cells represent the average of at least duplicate wells. Error is expressed as the standard deviation of the mean.

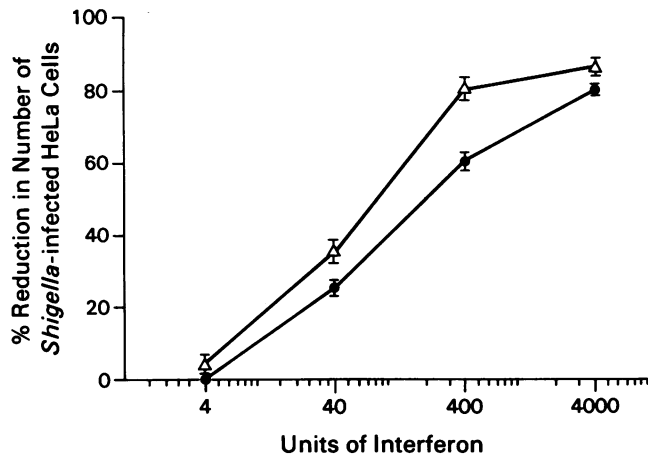


FIG. 3. Comparison of the abilities of nIFN- $\alpha$  (●) and nIFN- $\gamma$  (△) to inhibit HeLa cell invasion by *S. flexneri*. HeLa cell monolayers were pretreated with the indicated levels of IFN for 16 h prior to challenge with *S. flexneri* SA100. IFN was removed 1 h before challenge, and monolayers were washed with RPMI 1640. *Shigella* sp. was allowed to invade for 60 min. Noninternalized *Shigella* sp. was counterselected with 50  $\mu$ g of kanamycin per ml for 2 h. Points represent the average of four wells. Error bars show the standard error at each point.

pretreatment of cell monolayers with IFN also inhibits subsequent invasion of *S. flexneri* or *S. sonnei*. Both natural and recombinant IFNs inhibited *Shigella* invasion. IFN-mediated inhibition of *Shigella* invasion was time and dose dependent. The direct role of IFN in the induction of a cellular state which was resistant to *Shigella* invasion was supported by the following results. (i) Recombinant IFNs mediated the effect. (ii) Specific antiserum to IFN negated the inhibitory effect. (iii) The ability of IFN to induce a resistant cell population was species specific. These results clearly indicate that IFN pretreatment of cells induces a cellular state which is resistant to invasion by *Shigella* spp.

The mechanism by which IFN induces a resistant cellular state which inhibits bacterial invasion is unknown. However, IFN has been shown to alter the physiology and gene

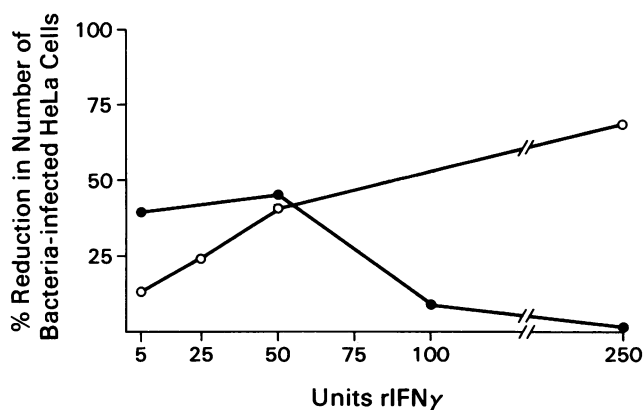


FIG. 4. Comparison of the inhibition of *Salmonella* (●) and *Shigella* spp. (○) by rIFN- $\gamma$ . HeLa cell monolayers were treated with the indicated concentrations of human rIFN- $\gamma$  for 16 h prior to challenge. Noninternalized bacteria were counterselected with 50  $\mu$ g of kanamycin per ml for 2 h prior to overlaying. Counts represent the average of at least duplicate wells.

TABLE 3. Inhibition of *Shigella* invasion of murine cell lines by murine nIFN

Target cell	Pretreatment <sup>a</sup>	Infected cell count (CFU/well) <sup>b</sup>	% Reduction in infected cells
Expt 1 MEF	Medium	268 $\pm$ 4	47
	1,500 U of IFN- $\alpha/\beta$	144 $\pm$ 4	
L cells	Medium	274 $\pm$ 27	49
	2,500 U of IFN- $\alpha/\beta$	141 $\pm$ 17	
Expt 2 MEF	Medium	98 $\pm$ 20	0
	1,000 U of human rIFN- $\gamma$	107 $\pm$ 27	
	1,000 U of murine IFN- $\gamma$	57 $\pm$ 5	

<sup>a</sup> Highly purified murine IFN- $\alpha/\beta$  was diluted in RPMI 1640 to produce the indicated concentrations per well. MEF and L-cell monolayers were treated for 20 h prior to bacterial challenge. No cytotoxicity to the monolayers was observed.

<sup>b</sup> Counts of infected MEF and L cells represent the average of at least four wells. Error is expressed as the standard deviation of the mean.

expression of eucaryotic cells dramatically, with many of these changes occurring at the cell membrane. Pfeffer et al. (32) and others (4, 5, 11, 42) have shown IFN-induced inhibition of cell motility, increased rigidity of plasma membranes, and a reduction in cell receptor movement. These membrane changes could affect bacterial adsorption to the cell surface or the actual penetration of eucaryotic cell membranes by the bacterium. The precise mechanism of inhibition is currently under investigation.

The production of soluble virulence-associated products by *Shigella* sp. may explain the loss of IFN-induced cellular resistance to bacterial invasion at high concentrations (MOI) of bacteria. Prizont (33) has shown that virulent strains of *Shigella* produce endoglycosidase activities which can alter eucaryotic cell surfaces. These alterations may lead to loss of IFN-induced surface changes and increased *Shigella* interaction at the cell surface. Alternatively, virulent *Shigella* strains have been shown to produce Shigalike toxin (17, 25). Shigalike toxin may alter the eucaryotic cell surface as a result of specific binding to its receptor on the cell surface (6) or through inhibition of protein synthesis (6, 34). Therefore, Shigalike toxin may alter IFN-induced cellular resistance by direct perturbation of the cell surface, thereby increasing the ability of *Shigella* sp. to interact with the cell, or by altered gene expression which may allow increased bacterial penetration.

Loss of the cellular IFN-induced resistant state with increasing MOIs or invasion times may explain the failure of previous investigators to observe inhibition of *Shigella* invasion by IFN. Using high MOIs and 2- to 3-h invasion times, which are necessary for microscopic detection of intracellular bacteria, Gober et al. (14) and Bukholm and Degré (7, 8) could not demonstrate inhibition of *Shigella* invasion. When similar conditions were used, IFN-induced inhibition of *Shigella* invasion was also not observed in our assay system (data not shown).

The mechanism(s) by which *Salmonella* and *Shigella* spp. are able to enter and replicate within nonphagocytic cells is unknown. However, on the basis of IFN-induced inhibition, it appears likely that distinctly different cellular binding sites or penetration mechanisms may exist for cellular invasion by

*Salmonella* versus *Shigella* spp. Experimental evidence for separate mechanisms are as follows. (i) The dose response of IFN-induced protection for *Shigella* sp. is linear from 0 to 1,000 U for both IFN- $\alpha$  and IFN- $\gamma$  (Fig. 1 and 2). With *Salmonella* sp., once a maximum inhibitory concentration was achieved (IFN- $\alpha$ , 50 U; IFN- $\gamma$ , 10 U), inhibition was reduced such that at higher IFN concentrations no protective effect was observed (Table 2) (7, 8). (ii) Establishment of an IFN-induced protective state was induced over many hours (Fig. 2) for *Shigella* sp. In contrast, inhibition of *Salmonella* invasion has been observed in monolayers to which *Salmonella* sp. and IFN were added concomitantly (8). The differential response of *Shigella* versus *Salmonella* spp. to IFN-pretreated cells suggests that these bacteria interact at different membrane sites or invade a cell via different mechanisms.

We clearly showed in this study that IFN pretreatment of epithelial cells inhibits *Shigella* invasion. This is an important consideration in host defense against facultative intracellular enteric bacteria, as noninvasive variants of these species have been demonstrated to be uniformly avirulent (12, 13, 35, 36). We are investigating IFN-induced inhibition of bacterial invasion for a number of invasive bacterial species as well as defining the role of IFN as a key element in host defense against invasive bacterial pathogens *in vivo*.

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