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Suppression of the Intracellular Growth of *Shigella flexneri* in Cell Cultures by Interferon Preparations and Polyinosinic-Polycytidylic Acid

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A strain of *Shigella flexneri* type 2a was found to multiply intracellularly in cultures of the diploid human cell strain FS-1 and in secondary rabbit kidney cells. When inoculated cultures were stained and observed under the light microscope, it appeared that the cytoplasm of infected cells became gradually filled with bacteria. Various preparations of human and rabbit interferon were found to suppress the intracellular bacterial growth in homologous cells. Polyinosinic-polycytidylic acid (poly I·poly C) had a similar inhibitory effect. Rabbit interferon preparations did not cause a significant suppression in human cells. Suppression of the bacterial growth could be demonstrated in two ways: (i) by showing that treatment with either homologous interferon or poly I·poly C reduced the proportion of infected cells determined by counting the total number of cells and the number of cells with 10 or more bacteria in several microscopic fields selected at random, or (ii) by showing that a suppression in ^{3}H-uridine incorporation by bacteria occurs in infected cultures treated with actinomycin D after incubation with interferon or poly I·poly C. (Uridine incorporation by the bacterium is insensitive to actinomycin D.) Treatment of cells with actinomycin D before incubation with interferon prevented the development of cellular resistance to bacterial infection. Interferon preparations did not have an inhibitory effect on the extracellular growth of *S. flexneri* in a broth culture. These findings show that the range of activity of the interferon system apparently extends to intracellularly growing bacteria.

Interferons are proteins which can render animal cells resistant to viral infection (19). Recent studies indicate that the intracellular multiplication of certain nonviral agents belonging to the families *Chlamydiaceae* (3, 5, 12, 18), *Protozoa* (4, 13), and *Rickettsiae* (6) is also inhibited by interferon and various interferon inducers (reviewed in reference 20). Weinstein et al. (22) found that the interferon inducer polyinosinic-polycytidylic acid (poly I·poly C) protected mice against various bacterial infections, but, since passive administration of interferon failed to protect animals, the authors concluded that interferon was probably not involved in this protection against extracellularly growing bacteria. Remington and Merigan (14) observed a similar protective effect of poly I·poly C in mice infected with *Listeria monocytogenes*.

In this communication, we show that various interferon preparations and the interferon inducer poly I·poly C suppress the intracellular multiplication of the *Shigella flexneri* in cultures of human and rabbit cells. This inhibitory effect on the growth of a bacterium shows characteristics that are in many respects similar to the antiviral action of interferon.

MATERIALS AND METHODS

Cell cultures and media. Two types of cell cultures were used. A diploid human cell strain, designated FS-1, was derived from newborn foreskin tissue. Rabbit kidney cells were obtained by trypsinization of fresh kidneys from weanling rabbits; secondary cultures were employed in the experiments. Cells were grown on glass cover slips (6 by 14 mm) placed in Leighton tubes in Eagle's minimal essential medium (MEM) supplemented with 10% heated fetal calf serum, 100 units of penicillin per ml, and 100 μg of streptomycin per ml. The medium was changed to antibiotic-free MEM 24 hr before inoculation with bacteria.

Bacteria. The virulent strain of *S. flexneri* type 2a

was kindly supplied by Joseph W. Winter, Beth Israel Hospital, New York, N.Y. The inoculum used for infecting cell cultures was obtained from a suspension of organisms grown in Brain Heart Infusion and Trypticase soy broth for 18 hr at 37 C. The bacterial cells were collected by centrifugation, washed, resuspended in antibiotic-free MEM, and diluted to 1 optical density unit at 500 nm (OD_{500}) per ml. Leighton tube cultures were infected with 1 ml of the bacterial inoculum per tube. Extracellular bacteria were removed by thorough washing with Earle's balanced salt solution 2 hr after inoculation and at 2-hr intervals thereafter. Throughout the experiment, cultures were incubated at 37 C in antibiotic-free MEM, supplemented with 2% fetal calf serum. Cover slips were removed from the tubes, washed with saline, fixed in methanol, stained with May-Grunwald, and counterstained with Giemsa. The proportion of infected cells in individual cultures was determined by counting the total number of cells and the number of cells containing 10 or more bacteria in several microscopic fields selected at random. To assure objectivity, in most experiments the person conducting the count was not familiar with the coding of cover slips.

Interferon preparations. Two preparations of human interferon were employed. One was prepared in this laboratory in human FS-1 cells with poly I·poly C as the inducer (Havell and Vilček, Bacteriol. Proc., p. 195, 1971). The other was produced in human leukocyte suspensions with Sendai virus as the inducer (17). The latter preparation was prepared by Kari Cantell and his colleagues in Helsinki, Finland, and supplied to us by Joseph A. Sonnabend, Mount Sinai School of Medicine, New York, N.Y. To remove infectious virus, the interferon preparation was dialyzed for 5 days against a *p*H 2 buffer at 4 C and subsequently centrifuged at 100,000 × *g* for 2 hr. Two different preparations of rabbit interferon were also used in these experiments. One was rabbit serum interferon prepared by intravenous inoculation of rabbits with Newcastle disease virus in the laboratory of Monto Ho, University of Pittsburgh. To remove residual virus, the serum was heated at 56 C for 1 hr and then centrifuged at 100,000 × *g* for 2 hr. (This preparation currently serves as the research standard for rabbit interferon.) The other interferon preparation was prepared in cultures of rabbit kidney cells with poly I·poly C as the inducer (21). Prior to use, all interferon preparations were dialyzed against two changes of antibiotic-free MEM. Interferon titrations were performed in human (17) or rabbit (21) cells by using plaque reduction assays with vesicular stomatitis virus. The potency of interferon preparations is expressed in standard units (11).

^{3}H-uridine uptake by cell cultures. Tube cultures, previously treated with interferon or untreated, were inoculated with the bacterial suspension as described above. At 7 hr after inoculation, actinomycin D (1 μg/ml) was added into each tube for 30 min at 37 C; the cells were then pulse labeled for 10 min with 10 μCi of ^{3}H-uridine. Thereafter, cultures were washed with saline and lysed in 1 ml of 0.01 M ethylenediaminetetraacetic acid-acetate buffer (*p*H 4.9) containing 0.5% sodium dodecyl sulfate. Material from this lysate, precipitated with 10% trichloroacetic acid, was collected on a membrane filter (Millipore Corp.), and radioactivity was counted in a liquid scintillation counter.

Chemicals used. Poly I·poly C was obtained from the Antiviral Substances Program, National Institute of Allergy and Infectious Diseases, Bethesda, Md. Diethylaminoethyl (DEAE) dextran (molecular weight about 2 × 10^6) was purchased from Pharmacia, Uppsala, Sweden. Uridine-*5-^{3}H* (20 Ci/mmole) and uridine-*5-^{3}H* (N) 5'-triphosphate (20 Ci/mmole) were obtained from New England Nuclear Corp., Boston, Mass. Actinomycin D was purchased from Calbiochem, Los Angeles, Calif.

RESULTS

Intracellular growth of S. flexneri. It has been suggested that cellular invasion and intracellular multiplication can be used as a criterion for virulence in the differentiation of strains of *S. flexneri* (1). We have observed intracellular multiplication of virulent *S. flexneri* type 2a in monolayers of FS-1 and rabbit kidney cells. When the cell cultures were examined under the light microscope 2 hr after inoculation with *S. flexneri*, few bacteria were observed in the infected cells. After 7 hr, practically the entire cytoplasm of infected cells became filled with bacteria (Fig. 1). The number of infected cells (i.e., cells containing 10 or more bacteria) in cultures at 7 hr after inoculation with 1 OD_{500} per ml of the bacterial suspension was usually approximately 5% of the total number of cells. The percentage of infected cells could be enhanced by using a larger bacterial inoculum or a longer incubation time at 37 C.

Effects of interferon and poly I·poly C on intracellular bacterial growth. Cover slip cultures of FS-1 cells in Leighton tubes were incubated for 18 hr with (i) human interferon, (ii) rabbit interferon, or (iii) the interferon inducer poly I·poly C in the presence of DEAE dextran. DEAE dextran was included because it is known to enhance the interferon-inducing capacity of poly I·poly C in FS-1 cells (Havell and Vilček, Bacteriol. Proc., p. 195, 1971).

The cultures were then thoroughly washed and inoculated with a suspension of *S. flexneri*. All cultures were fixed and stained 7 hr after inoculation. The number of infected cells in cultures treated with human interferon or poly I·poly C was reduced by about 80%. Treatment with heterologous rabbit interferon did not cause a significant reduction in the proportion of infected cells (Table 1). In control experiments, we found that the preparations of rabbit interferon employed did not suppress the growth of vesicular stomatitis virus in human cells.

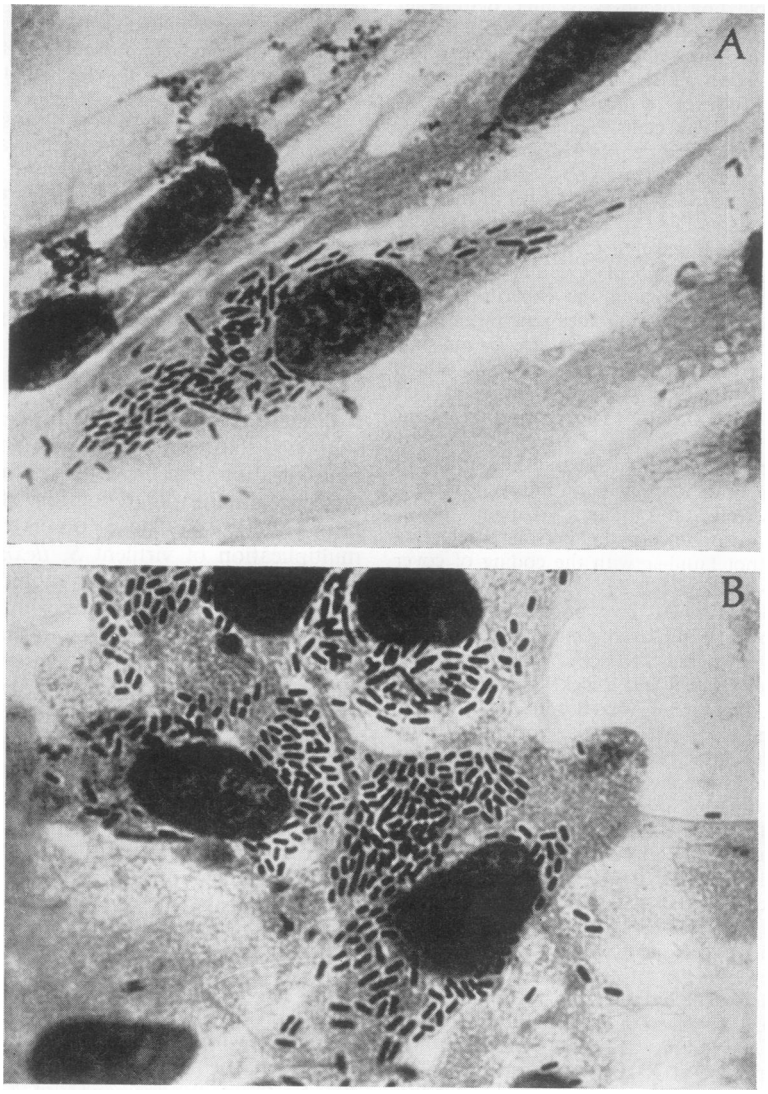

FIG. 1. *Microscopic appearance of FS-1 cells (A) and rabbit kidney cells (B) 7 hr after inoculation with S flexneri type 2a. Stained with May-Grunwald and Giemsa* (× 500).

^{3}H-uridine incorporation by actinomycin D-treated cells as a measure of intracellular bacterial growth and its supression by interferon. Microscope examination of cultures and the counting of infected cells proved a somewhat cumbersome method to measure the inhibitory effect of interferon on the intracellular growth of *S. flexneri*. A more simple and objective means of measurement was therefore devised, based on the incorporation of ^{3}H-uridine by cells infected with the bacteria. Concentrations of 1 μg of actinomycin D per ml inhibited ribonucleic acid (RNA) synthesis in both FS-1 and rabbit kidney cells by over 90%. *S. flexneri*, on the other hand, proved to be completely resistant to the action of actinomycin D (Fig. 2). It was thus possible to treat *S. flexneri*-infected cultures with actinomycin D before adding ^{3}H-uridine; the uptake of labeled uridine by the infected cultures minus uptake by uninfected actinomycin D-treated cultures reflected only the uptake due to the bacteria, thus providing a direct measure of bacterial growth in infected cell cultures. Care was taken to remove all extracellular bacteria by thoroughly washing the cover slips before incubating them with labeled uridine.

The effect of various concentrations of interferon on ^{3}H-uridine incorporation by intra-

TABLE 1. *Effect of various interferon preparations and of poly I·poly C on the growth of S. flexneri in human FS-1 cells*

Treatment	Expt 1			Expt 2			Expt 3		
	No. of cells infected/total no. counted	Cells infected (%)	P^a	No. of cells infected/total no. counted	Cells infected (%)	P^a	No. of cells infected/total no. counted	Cells infected (%)	P^a
None	19/373	5.0		25/382	6.5		25/507	4.9	
Human interferon[b]									
400 units/ml	2/313	0.6	<0.005	2/482	0.4	<0.005	5/444	1.1	<0.005
1,000 units/ml									
Rabbit interferon[c]									
400 units/ml	21/497	4.2	>0.05						
1,000 units/ml				16/466	3.4	>0.05	18/416	4.3	>0.05
Poly I·poly C[d]	3/308	0.9	<0.005	3/370	0.8	<0.005	5/522	0.9	<0.005

[a] Calculated from the χ^2 test.

[b] Interferon produced in FS-1 cells was employed in experiment 1, and human leukocyte interferon was used in 2 and 3. Cultures were incubated with interferon for 18 hr prior to inoculation with *S. flexneri*.

[c] Rabbit serum interferon employed in experiment 1, rabbit kidney cell culture interferon in 2 and 3. Cultures were incubated with interferon for 18 hr prior to inoculation.

[d] Cultures were treated with the poly I·poly C-diethylaminoethyl dextran complex (10 μg/ml and 50 μg/ml, respectively) for 1 hr, washed, and incubated in antibiotic-free media for an additional 17 hr prior to inoculation with *S. flexneri*.

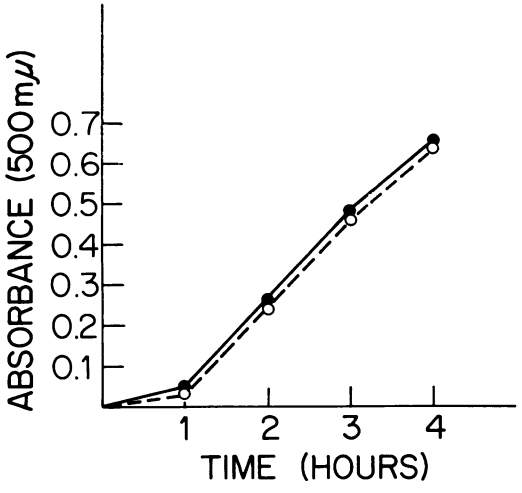

FIG. 2. *Growth curve of S. flexneri type 2a in nutrient broth with 1 μg of actinomycin D per ml (●) or with no actinomycin D (○).*

cellular bacteria is shown in Fig. 3. As few as 10 units of either human or rabbit interferon per ml was sufficient to produce a reduction in incorporation by the intracellular bacteria grown in FS-1 or rabbit kidney cells, respectively. A maximum inhibitory effect was achieved at a concentration of 100 units/ml in both types of cell culture; a further increase in the interferon concentration did not result in greater inhibition.

Lack of direct inhibitory effect of interferon on S. flexneri. A preparation of human interferon which was earlier shown to suppress bacterial growth in cultures of FS-1 cells showed no inhibitory effect on the extracellular multiplication of the same bacteria in a broth culture (Fig. 4). Furthermore, the inhibitory action of interferon on the intracellular bacteria was suppressed if cell cultures were treated with actinomycin D before their exposure to interferon (Table 2, group 5). These findings indicate that interferon does not cause direct inactivation of *S. flexneri* and that the suppression of intracellular bacterial growth by interferon requires deoxyribonucleic acid-dependent RNA synthesis.

A similar suppression of ^{3}H-uridine incorporation by intracellular *S. flexneri* was observed in cells exposed to the interferon inducer poly I·poly C (Table 2, group 3).

DISCUSSION

LaBrec et al. (7) showed that virulent strains of dysentery bacilli have the ability to penetrate epithelial cells of the intestine and to infect and multiply within cultured HeLa cells. Avirulent variants possessed neither of these capacities. The authors suggested that epithelial cell penetration is a major factor in determining the

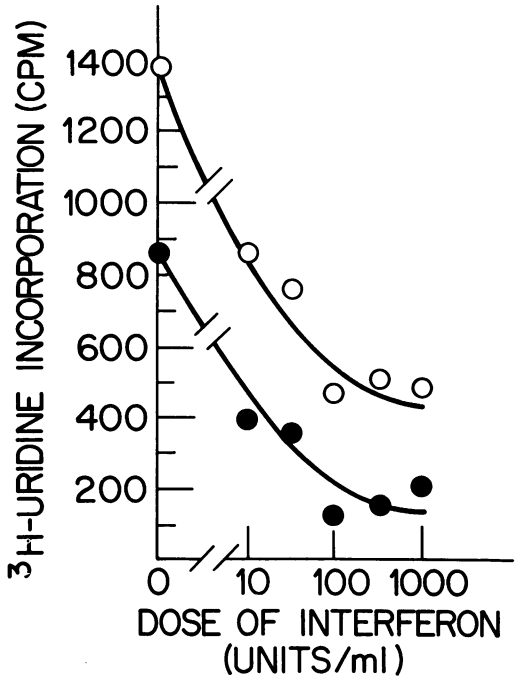

FIG. 3. *^{3}H-uridine incorporation in S. flexneri-infected cells treated with various interferon concentrations. FS-1 and rabbit kidney cell cultures, grown in screw-cap tubes without cover slips, were incubated for 18 hr with different concentrations of human leukocyte (○) or rabbit tissue culture (●) interferon, respectively. The cultures were then washed and inoculated with S. flexneri. Seven hours after inoculation, cells were treated with 1 μg of actinomycin D per ml for 30 min and pulse labeled for 10 min with 10 μCi of ^{3}H-uridine. Results are average counts per minute per tube minus counts per minute of control uninfected actinomycin D-treated cultures.*

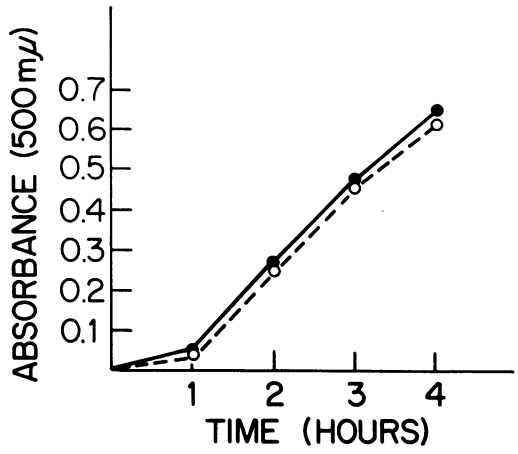

FIG. 4. *Growth curve of S. flexneri type 2a in nutrient broth with 250 units of human leukocyte interferon per ml (●) or with no interferon (○).*

TABLE 2. *^{3}H-uridine incorporation in S. flexneri-infected rabbit kidney cell cultures: effect of poly I·poly C and interferon*[a]

Group no.	Treatment	^{3}H-uridine incorporation (counts/min)	
		Expt 1	Expt 2
1	Uninfected, control	206	320
2	Infected, control	5,763	15,510
3	Poly I·poly C	ND[b]	3,290
4	Interferon	1,951	ND
5	Actinomycin D, then interferon	5,169	ND
6	Actinomycin D, no interferon	6,058	ND

[a] All cultures were treated with 1 μg of actinomycin D per ml 30 min before pulse labeling with ^{3}H-uridine as described in Fig. 3. All cultures, with the exception of group 1, were inoculated with *S. flexneri* 7 hr before labeling. Additional treatment was as follows: group 3 received 50 μg of poly I·poly C per ml 18 hr prior to inoculation. Groups 4 and 5 received 1,000 units of rabbit tissue culture interferon per ml 18 hr before inoculation. Group 5 was also exposed to 0.5 μg of actinomycin D per ml 30 min before the addition of interferon, and group 6 received identical treatment as group 5 but no interferon.

[b] ND, not done.

pathogenecity of dysentery bacilli. Intracellular multiplication of *S. flexneri* in HeLa cells was also demonstrated by Calabi (1). By using a microscopic technique similar to that described in these studies, it was possible to examine the penetration and intracellular growth of a pathogenic strain of *S. flexneri* type 2a in both human FS-1 and rabbit kidney cells.

In addition to the visual counting method, a more objective means was devised to measure the intracellular bacterial growth, based on the incorporation of labeled uridine into RNA. Actinomycin D inhibits RNA synthesis in FS-1 and rabbit cells but is ineffective in *S. flexneri*. By treating *S. flexneri*-infected animal cells with actinomycin D, it was possible to suppress selectively RNA synthesis in the animal cells. ^{3}H-uridine incorporation in such cultures could be employed as a measure of RNA synthesis by the bacterium as well as a measure of the inhibitory effect of interferon on bacterial growth.

It has been shown that phosphorylation of nucleosides occurs quite readily inside the cultured chick fibroblasts, with uridine being con-

verted into uridine triphosphate (UTP) (15, 16). When ^{3}H-uridine or ^{3}H-UTP was added to an *S. flexneri* broth culture, incorporation was demonstrated with both precursors although the rate of UTP incorporation was lower than that of uridine (L. L. Gober, *unpublished data*). It remains to establish what proportion, if any, of the ^{3}H-label added to infected cultures as ^{3}H-uridine was taken up by the bacteria in the form of UTP. However, the uncertainty as to whether uridine is converted to UTP prior to its uptake by intracellular bacteria does not affect the interpretation of our results.

There is evidence to suggest that the active components in the interferon preparations which inhibit intracellular bacterial growth and cause the antiviral effect are likely to be identical: (i) similar resistance of both activities to *p*H 2 treatment and heat, (ii) the fact that the continuous presence of the interferon preparation in the cultures was not required for either the antibacterial or antiviral action, (iii) the apparent correlation of the relative antibacterial and antiviral potencies of the four treated interferon preparations used in these studies. To provide definite evidence that the antibacterial effect and the antiviral activity in the interferon preparations are in fact due to the same component, further careful analysis will be necessary.

The antiviral action of interferon is believed to be mediated by a cellular antiviral protein. This hypothetical protein, produced in cells exposed to the action of interferon, is apparently responsible for the development of cellular resistance to virus infection (reviewed in reference 19). Three lines of evidence suggest that the suppression of *S. flexneri* multiplication by interferon is also indirect.

(i) Rabbit interferon preparations were ineffective in suppressing bacterial growth in human cells (Table 1) but were active when tested against the same organism in rabbit cells (Fig. 3). The lack of inhibitory action of rabbit interferon in human cells is in accord with the known tissue specificity of the antiviral action of interferon (19).

(ii) Interferon preparations which suppressed bacterial growth in cell culture showed no inhibitory effect on the extracellular multiplication of the same bacterium in a broth culture.

(iii) The inhibitory action of interferon on intracellular *S. flexneri* was suppressed if cultures were treated with actinomycin D before their exposure to interferon.

It is possible that the inhibitory effect of interferon on intracellular bacterial growth, like its antiviral action, is mediated by one or more newly synthesized cellular proteins. If so, then it is unclear whether this might be the same protein(s) that brings about the resistance to virus infection and whether this effect is related to the observed inhibitory effect of interferon and its inducers on the rate of cell division (2, 8). It is also not known which stage of the intracellular bacterial growth is affected by interferon. Contrary to earlier hypotheses (9, 10), it now seems unlikely that the host cell ribosome is the only target of interferon action, since bacteria contain their own functional ribosomes.

This study demonstrated for the first time the suppression of the growth of an intracellular bacterium by interferon preparations. Studies are in progress to determine whether the intracellular multiplication of some other bacteria is also inhibited.

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