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Interferon-mediated inhibition of virus penetration

(endocytosis/vesicular stomatitis virus/human, mouse, and chicken fibroblasts)

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ABSTRACT **Pretreatment of mouse L cells with mouse interferon (IFN) inhibits the penetration of vesicular stomatitis virus without affecting viral adsorption. The inhibition of virus uptake by IFN is dose dependent and, at the highest dose tested (1,000 units/ml), reaches 65%; 24 hr of treatment with IFN are required for maximal effect. A similar inhibition of uptake of virus occurs in human diploid fibroblasts and primary chicken embryo fibroblasts treated with homologous IFN. No significant inhibition occurs when cells are treated with heterologous IFN. These results document a previously unrecognized antiviral effect of IFN—namely, inhibition at the level of viral uptake.**

It has been generally accepted that in cells pretreated with interferon (IFN), virus growth is blocked at the level of gene expression (reviewed in ref. 1). In addition, there is evidence that IFN may block the maturation and release of certain viruses (2–5). Because several studies showed that IFN pretreatment can inhibit replication of infectious viral RNA but does not alter the ability of virus to adsorb to host cells, it has been assumed that IFN has no effect on the ability of virus to enter cells (6–12).

Virus uptake by animal cells is a two-step process: (*i*) adsorption of the virus to the cell surface, a temperature-independent function, and (*ii*) penetration or internalization of the virus, which occurs only at physiological temperatures (13, 14). It is known that some viruses carry out this second step by direct fusion of the virus and cell membranes, whereas other viruses are internalized by endocytosis (15–18).

In the course of studies on the effect of virus infection on endocytosis, we found that IFN inhibited endocytosis by cells, as measured by the uptake of a fluid-phase marker enzyme, horseradish peroxidase (unpublished results). Because endocytosis is believed to be the mechanism of penetration for many viruses, we tested the effect of IFN on the entry of vesicular stomatitis virus (VSV) into host cells and found that pretreatment of cells with IFN resulted in a marked inhibition of the uptake of VSV.

MATERIALS AND METHODS

Chemicals. [^{35}S]Methionine (1,180 Ci/mmol; 1 Ci = 3.7 × 10^{10} Bq) was obtained from Amersham. Actinomycin D was obtained from Sigma.

Cells and Virus. Mouse L cells and primary chicken embryo fibroblasts were cultivated at 37°C in 95% air/5% CO_2 in Eagle's minimal essential medium supplemented with 4% calf serum and 100 units of penicillin and 100 μg of streptomycin per ml (19). Human diploid fibroblasts were obtained from John Armstrong (University of Pittsburgh) and were cultivated in the same manner as chicken fibroblasts except that 4% fetal calf serum was used.

Stocks of VSV (Indiana strain) were grown in baby hamster kidney BHK-21 cells as described (20). Plaque assays of VSV infectivity were done in primary chicken embryo fibroblasts.

IFN. Crude mouse IFN (16,000 units/ml) was prepared in L cells infected with Newcastle disease virus as described (21). Purified human IFN-α, human IFN-β, and a mouse IFN-α/β mixture were obtained from Lee Biomolecular Laboratories (San Diego, CA); international units/mg of protein were: mouse IFN-α/β mixture, 2.7 × 10^5; human IFN-α, 2.3 × 10^5, and human IFN-β, 1.1 × 10^5. Crude chicken IFN (2,400 units/ml) was prepared in eggs infected with the WSN strain of influenza virus as described (22).

Preparation of [^{35}S]Methionine-Labeled VSV. VSV was grown in BHK-21 cells in methionine-free medium containing actinomycin D (5 μg/ml) and [^{35}S]methionine (50 μCi/ml). After 24 hr, the virus was pelleted from the medium and purified by two cycles of sucrose gradient centrifugation as described (20). ^{35}S-Labeled VSV purified in this manner had a titer of 4.2 × 10^{11} plaque-forming units (pfu)/ml, a specific activity of 6.5 × 10^9 cpm/ml, and a pfu/particle ratio of 0.06. When this preparation was examined by $NaDodSO_4$/polyacrylamide gel electrophoresis, only the five VSV proteins were detected (see Fig. 2).

Virus Penetration. Monolayer cultures of L cells in 35-mm dishes (3–6 × 10^5 cells) were incubated for 60 min at 37°C with 0.5 ml of complete medium containing ^{35}S-labeled virus at the multiplicity of infection (moi) indicated, usually 5. In each experiment, an equal number of mock-treated and IFN-treated cells were infected. After incubation, the inoculum was removed and the cells were washed twice with 1 ml of Hepes/saline (10 mM Hepes, pH 7.2/150 mM NaCl). Then the monolayer was incubated with 1 ml of standard trypsin/EDTA (0.25% trypsin (Difco)/0.025% EDTA in phosphate-buffered saline) for 7 min at room temperature to remove virus adsorbed to the cell surface. To terminate the trypsin treatment, 1 ml of Hepes/saline containing 10% calf serum was added, the cells were harvested and centrifuged at 2,000 rpm for 5 min in an International model PR-2 centrifuge (rotor 269). The pellet was washed once with 1 ml of Hepes/saline/10% calf serum, and the final pellet was solubilized in 1 ml of Hepes/saline containing 0.5% $NaDodSO_4$. The radioactivity in the cell extract was determined by liquid scintillation counting. The protein concentrations of the final solubilized cell pellets were determined by the method of Lowry *et al.* (23). The solubilized cell pellets of mock-treated and IFN-treated cells did not differ significantly in their protein concentrations. The amount of radioactive virus taken up by cells was expressed as counts per min per mg of protein.

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Abbreviations: IFN, interferon; VSV, vesicular stomatitis virus; moi, multiplicity of infection; pfu, plaque-forming unit.

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Virus Adsorption. L-cell monolayers were precooled to 4°C for 15 min, infected with ^{35}S-labeled VSV at a moi of 5, and incubated for 60 min at 4°C. Unadsorbed virus was removed by three washes with Hepes/saline precooled to 4°C, and the cells were solubilized in Hepes/saline/0.5% $NaDodSO_4$. The amount of cell-associated radioactivity was determined by liquid scintillation counting. Protein was determined in each sample, and all values for radioactivity were normalized to a protein concentration of 1 mg/ml. At least 99% of the radioactive virus that was cell-associated under these conditions was sensitive to removal by trypsin treatment.

RESULTS

The Effect of IFN on VSV Penetration. In order to test the effect of IFN on VSV penetration, L-cell monolayers were pretreated for 24 hr at 37°C with 1,000 units of crude mouse IFN. The IFN-containing medium was removed, and the cells were infected with purified ^{35}S-labeled VSV (moi, 5). At the indicated times after the addition of virus, the amount of trypsin-insensitive ^{35}S-labeled VSV associated with the cell was determined. IFN pretreatment of L cells significantly reduced the rate of virus uptake as compared to mock-treated cells (Fig. 1). At the end of 2 hr of incubation with virus, the control cells had internalized 12.2% of radioactive virus contained in the inoculum, and the IFN-treated cells had taken up only 5.0% of the inoculum.

The radioactive virus that was cell-associated after trypsin treatment was examined by $NaDodSO_4$/polyacrylamide gel electrophoresis. A normal complement of the five VSV proteins was internalized by both control and IFN-treated cells (Fig. 2, lanes 2 and 3).

The IFN-mediated inhibition of virus uptake was dose dependent (Fig. 3). Cells were mock-treated or treated with purified or crude IFN (1–1,000 units/ml) for 24 hr at 37°C. Although IFN at 1 unit/ml had no effect, at 10 units/ml it inhibited virus uptake by 7% and at 1,000 units/ml it inhibited virus penetration by >65%. This dose–response was similar to the level of inhibition observed when fluid-phase endocytosis was measured by the uptake of a marker enzyme, horseradish peroxidase (unpublished results). There was no significant difference between the activity of the purified and crude IFN prepara-

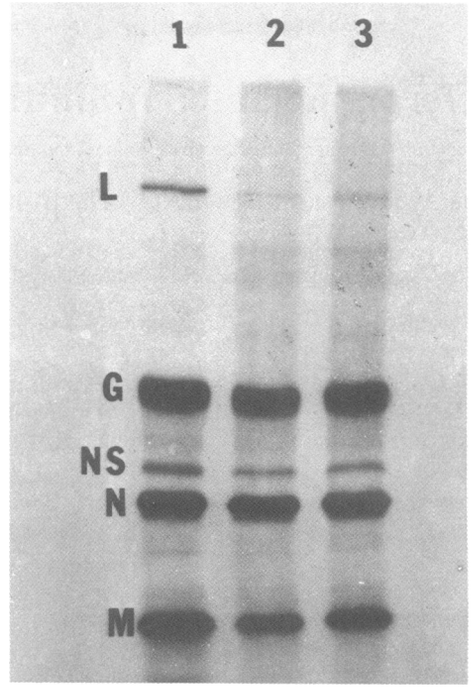

FIG. 2. Analysis of ^{35}S-labeled VSV protein taken up by IFN-treated and control L cells. Cell monolayers were treated with IFN as described in the legend to Fig. 1. Cells were then infected with ^{35}S-labeled VSV at an moi of 20. After 60 min of incubation at 37°C, the trypsin-insensitive cell-associated radioactivity was prepared and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis by the method of Laemmli (24) as modified by Lesnaw (25). Lanes: 1, ^{35}S-labeled VSV inoculum; 2, ^{35}S-labeled VSV proteins internalized by control cells; 3, ^{35}S-labeled VSV proteins internalized by IFN-treated cells. Equal amounts of radioactivity were used for each lane. L, G, NS, N, and M indicate the migration positions of the five VSV proteins.

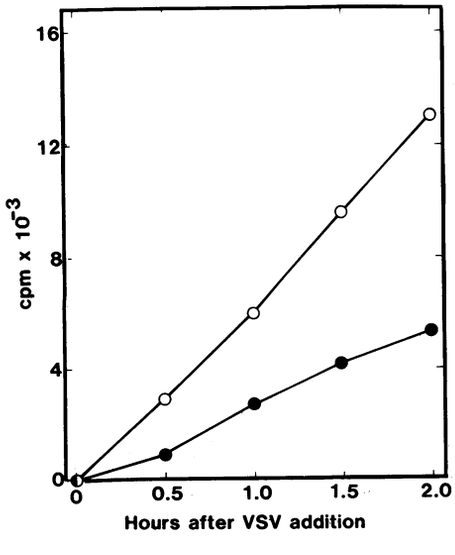

FIG. 1. Effect of treatment with IFN on uptake of VSV by mouse L cells. Cell monolayers were mock-treated or treated with crude mouse IFN at 1,000 units/ml for 24 hr and infected with ^{35}S-labeled VSV. At the indicated times after infection, the amount of trypsin-insensitive cell-associated radioactivity was determined. Each experimental point is an average of three separate determinations. ○, Mock-treated cells; ●, IFN-treated cells.

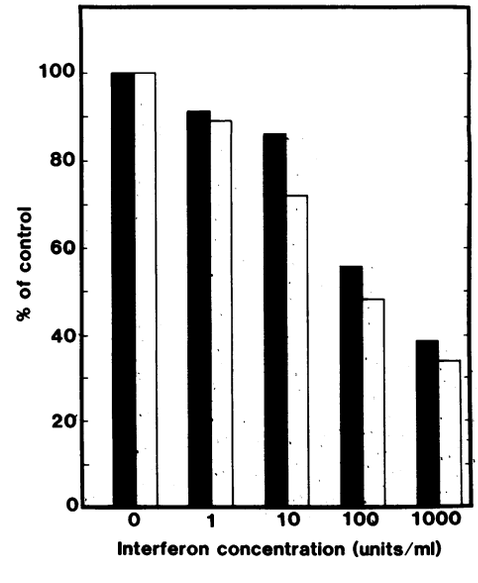

FIG. 3. Effect of IFN concentration on the uptake of VSV by mouse L cells. Cell monolayers were treated with the indicated concentrations of IFN for 24 hr at 37°C. The cells were infected with ^{35}S-labeled VSV, and the amount of virus internalized after 1 hr at 37°C was determined. Each bar represents an average of three experiments. The control value representing maximum (100%) virus uptake was 10,200 cpm per mg of cell protein. □, Crude mouse IFN; ■, purified mouse IFN.

Table 1. Lack of effect of IFN treatment on adsorption of VSV to mouse L cells at 4°C

Cells	^{32}S-Labeled VSV, cpm		
	Input	Bound*	Trypsin insensitive†
Untreated controls	9.7×10^5	1.1×10^4	3.2×10^1
IFN-treated‡	9.7×10^5	1.6×10^4	8.6×10^1

*Radioactivity bound after 60 min at 4°C.
†Trypsin-insensitive radioactivity bound at 4°C.
‡Cells treated with IFN at 1,000 units/ml for 24 hr at 37°C.

tions. Furthermore, the percentage inhibition of VSV uptake was independent of the moi: at 20, 2, and 0.2 pfu per cell, virus penetration was inhibited to a similar extent by IFN at 1,000 units/ml (data not shown).

To test the possibility that the reduced penetration of VSV into IFN-treated cells was due to a blockage of viral adsorption, the adsorption of VSV was measured in mock-treated and IFN-treated L cells. There was no significant difference in the amount of ^{35}S-labeled VSV bound at 4°C to control or to L cells pretreated with 1,000 units of IFN per ml. In fact, IFN-treated cells adsorbed slightly more radioactive virus than did control cells (Table 1). It should be noted that when cells were infected at 4°C, nearly all adsorbed virus particles remained sensitive to removal by trypsin treatment (Table 1).

Specificity of IFN Action on Penetration. The inhibition by IFN was species specific (Table 2). Mouse IFN had no effect on the uptake of ^{35}S-labeled VSV by human and chicken cells. However, the blockage of VSV penetration occurred in human cells treated with human IFN-α and in chicken cells treated with chicken IFN (Table 2). Similar results were obtained in human cells treated with human IFN-β (data not shown). Human IFN-α had a slight inhibitory effect in mouse L cells; this finding is consistent with reports of a limited antiviral effect of human IFN on mouse cells (26).

Induction and Decay of IFN-Mediated Inhibition of Viral Penetration. The full expression of IFN-mediated inhibition of VSV penetration required several hours of IFN treatment (Fig. 4). Maximal inhibition was observed after 24 hr of treatment with 1,000 units of IFN per ml at 37°C. The kinetics of induction of this effect are similar to the kinetics of development of the antiviral state in L cells (1). However, a direct kinetic comparison is complicated by the fact that virus uptake is measured over a short time period (1 hr), whereas measurement of the antiviral state requires a complete virus growth cycle. It is difficult to establish whether or not the IFN-induced inhibition of VSV penetration requires *de novo* host cell gene expression, because inhibitors of host macromolecular synthesis such as cycloheximide or actinomycin D also inhibit endocytosis after 6–8 hr of treatment (unpublished observation).

Table 2. Species specificity of IFN-mediated inhibition of VSV penetration

IFN species*	% inhibition of VSV penetration†		
	Mouse cells	Human cells	Chicken cells
Mouse (purified)	65	0	0
Human (α)	10	64	ND
Chicken	4	ND	66

ND, not determined.
*Cells were treated with IFN at 1,000 units/ml for 24 hr.
†The uptake of ^{35}S-labeled VSV in mock-treated control cells after 60 min at 37°C was 9.8×10^3, 6.4×10^3, and 1.1×10^4 cpm/mg of cell protein for mouse, human, and chicken embryo cells, respectively.

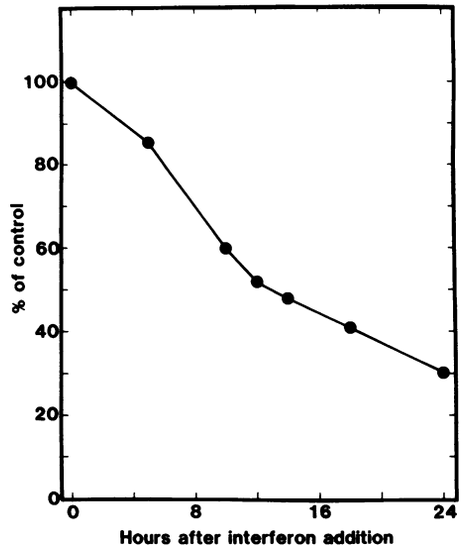

FIG. 4. The kinetics of induction of the IFN-mediated inhibition of VSV uptake. Monolayers of L cells were treated with IFN at 1,000 units/ml. At the indicated times after the addition of IFN, the cells were infected with ^{35}S-labeled VSV, and the amount of trypsin-insensitive virus that was cell-associated after 1 hr at 37°C was determined. The control value representing maximum (100%) virus uptake was 6,100 cpm per mg of cell protein.

In order to study the decay of the IFN effect on virus uptake, monolayer cultures of L cells were treated with crude mouse IFN at 1,000 units/ml for 24 hr at 37°C. The IFN-containing medium was removed and replaced with fresh medium. The uptake of ^{35}S-labeled VSV was determined at 24-hr intervals. Removal of the IFN from cells resulted in a decay of the inhibition of virus uptake (Fig. 5). By 72 hr after IFN removal, the cells that had been treated with IFN internalized virus at the same rate as did control cells.

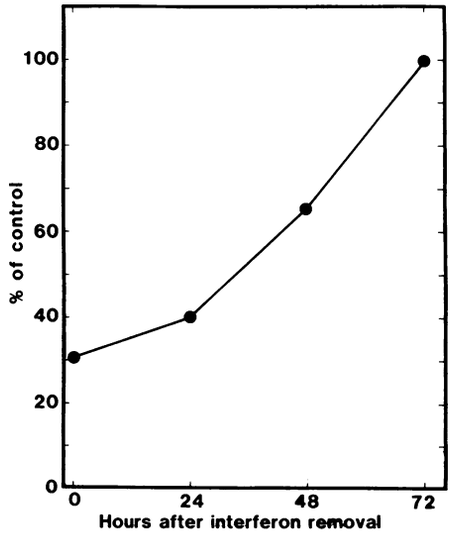

FIG. 5. Rate of decay of IFN-mediated inhibition of VSV uptake. Monolayers of L cells were treated with IFN at 1,000 units/ml for 24 hr. At time zero, the IFN was removed and the cells were washed once with complete medium. At the indicated times after IFN removal, the cells were infected with ^{35}S-labeled VSV, and the amount of trypsin-insensitive virus that was cell-associated after 1 hr at 37°C was determined. The control value representing maximum (100%) virus uptake was 9,730 cpm per mg of cell protein.

DISCUSSION

Although the IFN-induced antiviral response is known to be multifaceted, it has been thought that IFN had no effect on the virus growth cycle at the level of penetration (1, 6, 10–12). The data in this paper show that IFN treatment markedly reduces the penetration of VSV into cells without affecting the adsorption of virus to the cell surface. The effect of IFN on viral penetration is dose dependent, and the kinetics of induction and decay of the IFN-mediated inhibition of uptake are similar to those observed for the induction and decay of the IFN-mediated antiviral response (1). The inhibition of VSV penetration by IFN is species specific and requires homologous IFN in mouse, human, and avian cells.

The substantial reduction of virus entry after IFN treatment could explain the inhibitory effect of IFN on early events in viral infection, such as primary transcription by VSV (27, 28). The observation that IFN reduces the amount of simian virus 40 DNA that enters the nuclei of infected cells (29) also may result from a reduced rate of virus uptake.

Because IFN inhibits endocytosis, it might be predicted that penetration of all viruses that enter the cell through endocytosis may be inhibited similarly. It will be of particular interest to determine if IFN can inhibit the uptake of viruses that enter the cell by direct membrane fusion rather than by endocytosis (18).

Beside antiviral effects, IFN is known to inhibit the growth of many other intracellular parasites, including *Rickettsiae*, some species of bacteria, and protozoa (30). Compared to the inhibition of virus, a much higher dose (10- to 100-fold) of IFN is needed to inhibit these infectious agents (31). These dose levels are similar to those shown here to inhibit virus uptake. Because endocytosis is known to be the mode of entry for many of these intracellular parasites (32–34), the IFN-mediated inhibition of endocytosis may be responsible, at least in part, for the inhibitory effects of IFN.

Compared to other known antiviral effects of IFN (e.g., inhibition of translation of viral proteins), the inhibition of virus penetration is somewhat less dramatic and probably only serves as a first line of defense. It requires comparatively high doses of IFN, but those dose levels are well within physiological limits. In the vicinity of IFN-producing cells *in vivo*, local IFN concentrations have been calculated to be as high as 10^7 units/ml (35). Although viral uptake is only partially blocked by IFN treatment, at low multiplicities of infection this level of inhibition could protect a significant proportion of a cell population from infection.

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