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Mode of Action and Biological Properties of Insoluble Interferon (38329)

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In a previous publication, we reported that mouse interferon covalently attached to Sepharose 4B beads induced an antiviral state in sensitive L cells (1). This antiviral effect was neutralized by serum specific to mouse interferon, but reappeared almost completely when the antibody was removed from the insoluble interferon by treatment at pH 2.3. In this publication, we will explore possible modifications of the mechanism of induction of the antiviral effect, and of the physicochemical properties that might have occurred after the binding of interferon to Sepharose beads.

Materials and Methods. Cell lines. Mouse L cells were routinely propagated in this laboratory.

Medium. The cells were grown in Eagle's medium with 10% calf serum. For maintenance of the cells, serum concentration was reduced to 2%.

Viruses. The Indiana strain of vesicular stomatitis virus (VSV) and encephalomyocarditis virus (EMC) were routinely passaged in our laboratory in L cells. Virus was titrated by plaque assays in L cells. Hemagglutinations of EMC were performed at 4°, using human group O erythrocytes.

Preparation and assay of mouse interferon. Mouse interferon was prepared in a clonal line of L cells, using Newcastle disease virus (NDV), Hertsfordshire strain, as an inducer. The interferon was concentrated 10-fold by pressure dialysis and assayed in L cells using VSV as the challenge virus. The titer is expressed as the dilution which inhibits 50% of the cytopathic effect of the challenge virus. After concentration, the usual titer was about 10^5 units per 0.1 ml.

Coupling of interferon to Sepharose. Crude or purified interferon was bound to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals AB, Uppsala, Sweden), using Porath's technique (2). The Sepharose was suspended in 500 ml of 1 m*M* HCl. The beads were allowed to settle and were washed with an equal volume of 1 m*M* HCl, followed by two washings with 300 ml of 0.1 *M* sodium bicarbonate buffer (pH 8) containing 0.5 *M* NaCl (buffer A). The packed Sepharose gel (17-ml gel volume) was suspended in 30 ml of an interferon solution (2×10^4 ref units per 0.1 ml) containing 0.6 mg of protein per 0.1 ml, which had been previously dialyzed for 18 hr at 4° against buffer A. The suspension was gently agitated at room temperature (24°) for 2 hr. After centrifugation, the supernatant was removed and the sedimented Sepharose beads were washed with 50 ml of buffer A. The beads were then incubated with 50 ml of 1 *M* ethanolamine (pH 8) for 90 min at room temperature with gentle stirring. Thereafter, the Sepharose beads were placed on the top of a 47-mm diam Millipore filter and washed as follows: twice with 80 ml of buffer A; twice with 80 ml of 0.1 *M* sodium acetate buffer, containing 1 *M* NaCl (pH 4.0) (buffer B); twice with 80 ml of Eagle's medium; once with 250 ml of Eagle's medium; once with 250 ml of 0.1 *M* sodium borate buffer, containing 1 *M* NaCl (pH 8.0) (buffer C); once again with 250 ml of buffer B; once with 250 ml of buffer C; and finally three times with 250 ml each of Eagle's medium.

In addition to the above-mentioned steps, in recent experiments, to remove interferon molecules simply absorbed in the Sepharose beads, the Sepharose was mounted in a column (10 cm height and 0.5 diam) and perfused with: (a) 50 ml of bovine albumin (2 mg protein/ml); (b) 500 ml of MEM medium. The amount of interferon eliminated was monitored using

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biological tests. At the end of the washings, no residual activity could be detected; the covalently linked interferon corresponded to about 90% or more of the biological activity, while an average of only about 50% of the contaminating proteins were coupled.

Experimental. Induction of the antiviral state by Sepharose-bound mouse interferon. Sepharose-bound (1.5×10^5 beads/10^6 cells) or soluble interferon (5×10^3 ref units/ml) were incubated for selected intervals with sensitive L cells grown in 35-mm plastic petri dishes. The interferon–Sepharose beads or soluble interferon were then removed. The cells were thoroughly washed with medium, and further incubated at 37° with medium for a total of 27 hr. They were then challenged with VSV at a multiplicity of infection (m.o.i.) = 0.1, and the viral yield was measured in L cells after 16 hr by routine plaque techniques.

As shown in Table I, both soluble and insoluble interferon produced a significant antiviral state within an hour. In both cases, the antiviral state increased with increasing contact periods of interferon and cells. For Sepharose-bound interferon, a maximum was reached after 5 hr; whereas for soluble interferon, the antiviral state continued to increase up to 27 hr.

It was of interest to explore the minimal number of beads per cell that was necessary to induce a maximal antiviral state. When observed with an optical microscope in phase contrast (as shown in Fig. 1), one bead covered by transparency an average of about 14 cells; however, because of its spherical shape, one bead probably touched one, or a very small number of cells at any given time. It is of importance to note that the beads did not attach strongly to the cell surface, but were rolling on the tissue culture sheet with the movement of currents in the culture medium.

TABLE I. Effect of Incubation Time on the Antiviral State Induced with Soluble or Sepharose-bound Interferon. The Results are Expressed as the Ratio of VSV Yield in Control/Treated Cells in Log 10.

Contact period (hr)	Antiviral state in cells treated with: Soluble interferon	Antiviral state in cells treated with: Sepharose-bound interferon
1	0.6	0.7
2	0.8	1
3	1.5	1
4	1.6	1.9
5	1.8	2.3
27	3.4	2.3

When the dose-response relationship between the number of interferon–Sepharose beads employed and the antiviral state was studied, the surprising observation was made that one bead per 40 cells was sufficient to induce maximal inhibition of the replication of EMC (1). Even one bead per 400 cells resulted in 99% inhibition of viral yield. When VSV, instead of EMC, was employed as the challenge virus, the number of beads that caused maximal inhibition was even smaller (unpublished data).

These results can be interpreted in two different ways: first, a small amount of interferon is detached from the beads in contact with the cells, and the solubilized antiviral material diffuses to neighboring cells. This antiviral material could be interferon itself, an active fragment therefrom, or the substance induced in the cells by interferon. Second, the interferon beads, themselves, which are able to move freely over the cell monolayer, could induce one or a series of modifications in the cells, which after multiple contacts between cells and beads, lead to a fully expressed antiviral state.

To explore these possibilities, free movement of the interferon–Sepharose beads was prevented in the following manner (see Fig. 2): a polyethylene ring was placed in the center of a 60-mm plastic petri dish containing a monolayer of mouse L cells. In one set of experiments, interferon–Sepharose beads (one bead/10 cells) were placed in the center of the ring, which kept them from escaping into the surrounding area (B). In another set, free interferon (8×10^4 ref units/ml) was added to the center of the ring (C). After 22 hr, the beads or free interferon were removed and about 400 infectious units of VSV were added. In the control not treated with interferon (A), about 390 plaques were counted, of which about 70 were found in the area corresponding to the interior of the ring used in B and C. In B, about 310 plaques were counted outside of the ring and only small, hardly visible microplaques inside of the ring. In B and C, the imprint of the ring is easily detectable. Thus, the antiviral material diffused outside of the ring when free interferon was used (C), but remained restricted to the interior of the ring when Sepharose-bound interferon was employed (B).

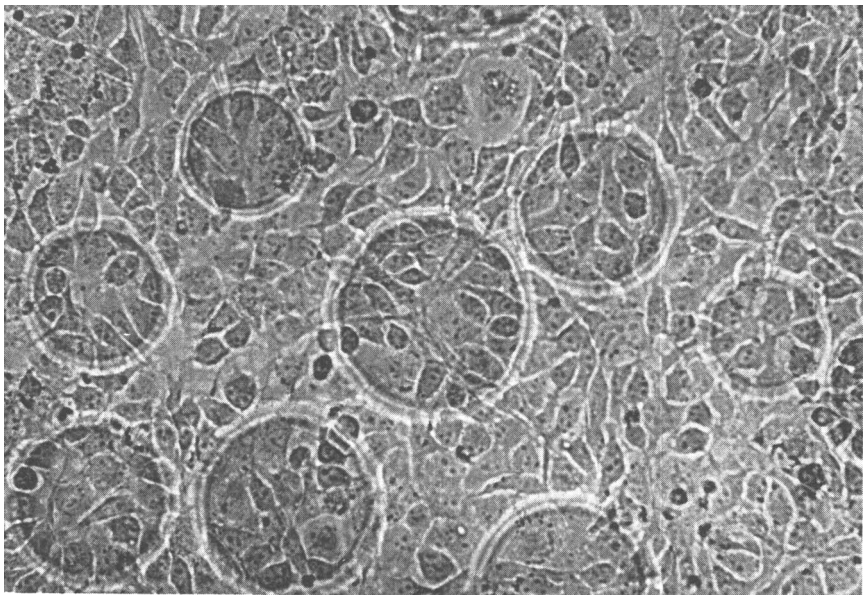

FIG. 1. Interferon–Sepharose beads partially covering mouse L cells in culture. Cell to bead ratio approximately 50:1 (phase contrast, magnification 160×.)

These experiments do not exclude the possibility, however, that small amounts of interferon (undetectable by the technique described above), or fragments from the molecule, could have been detached during contact with the cells. If this were the case, it would be expected that after serial passages, the antiviral effect of the beads should progressively decrease and eventually disappear.

In previous experiments, we have shown that after four cell-to-cell transfers of one bead/20 cells, no decrease in the antiviral effect occurred (1). In a new series of experiments, similar results were obtained after six cell-to-cell transfers, even when the bead/cell ratio was reduced to 1/100. No detectable amount of interferon was found either in the supernatant of control interferon beads kept for 5 days at 37° or in the supernatant assayed at each passage level (unpublished data).

It, therefore, appears likely that the Sepharose-bound interferon induces the antiviral state by simple cell contact and that full protection of the cells can only be obtained when the beads are freely moving on the cell surface, due to Brownian movement and/or convection caused by small variations of temperature in the incubator. To test this hypothesis, decreasing numbers of interferon–Sepharose beads were incubated with 10^6 L cells. One set was incubated in a stationary position for 24 hr; another set was gently agitated with balancing movements. The beads were then removed and the cells challenged using EMC virus at a m. o. i. = 1. As summarized in Table II, slow agitation produced a significant antiviral state when the number of beads was so small that practically no protection was obtained under stationary conditions. The effect was only detected when the viral yield was estimated by plaque assays.

It was of interest to explore the possible modifications which could have occurred in the dose-response relationship between the amount of interferon fixed on the beads and the antiviral state. In the case of soluble interferon, the dose-response curve is sigmoidal. This was interpreted previously as relating to a cooperative effect between the receptor site of interferon, located in the cell membrane, and an activator site (3). Interferon, in decreasing 2-fold concentrations was fixed on activated Sepharose 4B beads, as described (15 ml of interferon/1.5 g Sepharose dry weight). Prior to fixation, the protein concentration of each suspension was adjusted (using purified bovine albumin) to 1.3 mg/ml. The beads (one bead/10 cells) were added to 10^6 L cells and incubated for 24 hr at 37°. The beads were then removed and the cells

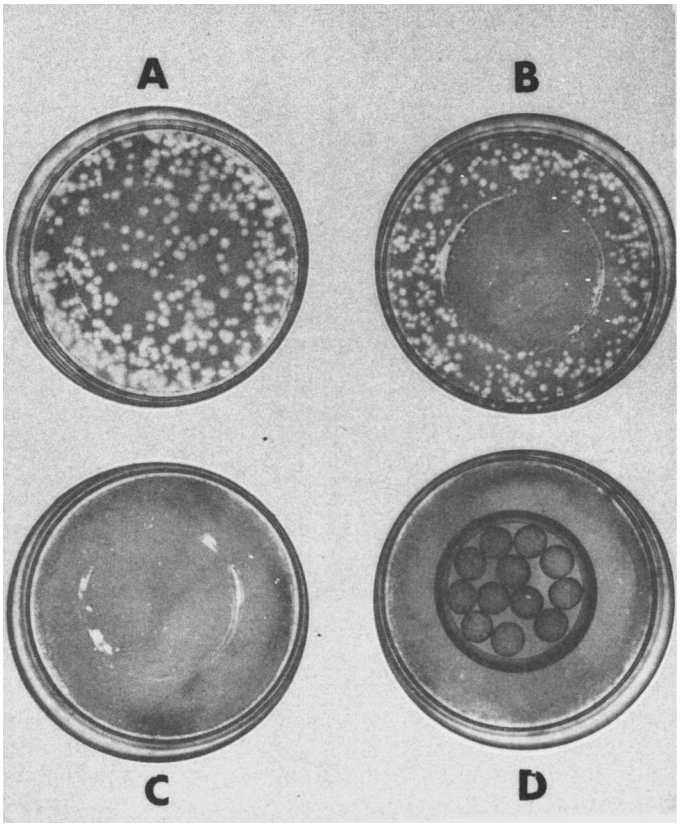

FIG. 2. Localized antiviral effect of Sepharose-bound interferon beads. The free movements of the beads are restricted inside an area limited by a plastic ring simply deposited on the tissue culture sheet. For the purpose of illustration, glass beads of distinguishable size, enclosed by the same type of plastic ring as used in B and C, are shown in D. In C, free interferon diffused under the ring inducing the antiviral state in the surrounding area. In B, protection is enclosed in an area clearly limited by the trace of the ring. In A, control virus.

washed and infected with VSV at a m. o. i. = 0.1 PFU/cell. Viral yield was assayed after a 16-hr incubation period at 37°. The dose-response curves obtained were sigmoidal, reminiscent of those observed with soluble interferon. Thus, no significant differences in the dose-response effect could be detected between soluble and Sepharose-bound interferon (3) (Fig. 3).

Physicochemical properties of Sepharose-bound interferon. Free interferon and interferon bound to Sepharose were autoclaved for 30 min at 110° (1 kg/cm^2 pressure) at different pH levels between pH 2 and pH 9. The results are summarized in Table III. Sepharose-bound interferon lost very little of its antiviral potency at pH 3; however, its biological activity gradually diminished at increasing pH values. Surprisingly, soluble mouse interferon was also quite resistant to heat at very low pH values, but at values from pH 5 to pH 7, the antiviral activity was completely destroyed.

The effect of crystalline pepsin and crystalline trypsin was also assayed (Table IV). Pepsin (Worthington Biochem. Corp.) was suspended in a sodium phosphate 0.2 *M*–citric acid 0.1 *M* buffer, adjusted to pH 3. The insoluble interferon (4 ml) was incubated with 1 ml of pepsin (200 μg/ml) at 37° for 1 hr. Then the pepsin was removed by centrifugation, the beads washed and assayed. Trypsin (Worthington Biochem. Corp.) (250 μg/ml), suspended in MEM medium, adjusted to pH 8, was incubated at 39° for 1 hr with the same amount of

TABLE II. Effect of Agitation on the Antiviral State Induced by Sepharose-Interferon. The Results are Expressed in Plaque-Forming (PFU) or in Hemagglutinating (HA) Units.

Number of cells/bead	With agitation		Without agitation	
	PFU	HA	PFU	HA
1000	4.7×10^7	1024/2048	3.3×10^8	2048
2000	4.5×10^7	≥2048	6×10^8	≥2048
Control	6.8×10^8	≥2048	6×10^8	≥2048

interferon–Sepharose beads. As control, a same volume of soluble interferon, titrating 10^5 ref units/ml, was similarly treated. In the case of soluble interferon, the effect of trypsin was blocked by Iniprol Choay (2.10^5 IP units/ml of interferon). Pepsin almost completely inactivated interferon–Sepharose; while the amount of trypsin which completely destroyed soluble interferon, was less effective in the case of Sepharose-bound interferon.

Discussion. Our experiments show that the biological properties of interferon covalently bound to Sepharose are fully expressed and perhaps even enhanced. The fact that a relatively small number of interferon–Sepharose beads is sufficient to block almost completely the replication of the virus could be due to small amounts of interferon that become separated from the beads during contact with the cells. It is also possible that only active fragments of the interferon molecule could be detached. A cell-to-cell spread of an interferon-induced antiviral protein is also among the possibilities.

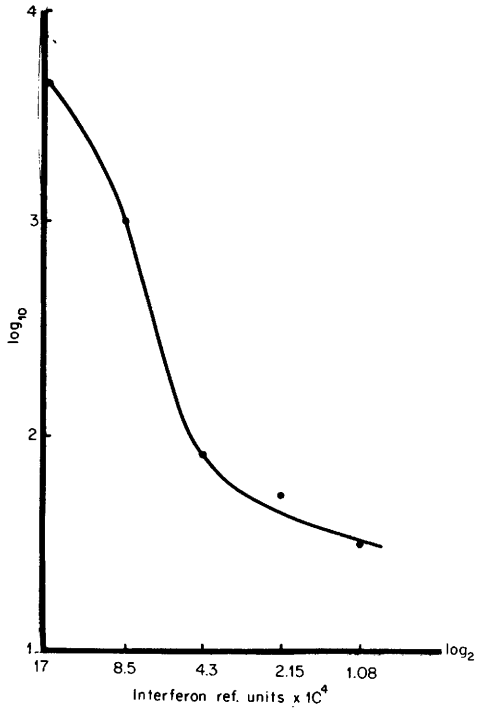

FIG. 3. Sigmoidal dose-response effect of serial concentrations of mouse interferon fixed on the same number of Sepharose beads (at each dilution 1 bead/10 cells is employed). The results are expressed as the ratio of VSV yield in control/treated cells in log 10.

An alternative hypothesis is that interferon induces the antiviral state solely by contact with the cell receptors, and that at every new contact the antiviral effect increases. This second hypothesis is substantiated by the lack of detectable antiviral activity diffusing out of a plastic ring used to restrict free movement of the Sepharose-bound interferon. The maintenance of unchanged antiviral properties of Sepharose-bound interferon after five 24-hr cell-to-cell transfers also supports this hypothesis. Since the dose-response relationship of soluble interferon is sigmoidal, it is unlikely that small amounts of interferon, which could become detached from the beads at each 24-hr period, would induce such a strong antiviral state. In addition, when serial concentrations of interferon are fixed to a constant amount of Sepharose beads and then incubated at maximal densities with sensitive cells, the dose-response curve obtained is equally sigmoidal.

Thus, our data are in agreement with the hypothesis that: (i) interferon acts merely by contact with cell membrane receptors without penetration into the cell; and (ii) a critical concentration of interferon molecules in contact with the cell receptors is required to induce the antiviral state by a yet unexplored cooperative mechanism. This view is also supported by findings by Kohno *et al.*, who postulated that the antiviral state developed in cells shortly after specific cell–interferon interactions (4). Similarly, Stewart suggested that, although interferon receptor interaction is necessary to the acti-

TABLE III. Effect of Autoclaving at Different pH Levels at 110° (1 kg/mm pressure) on the Antiviral Properties of Sepharose-Bound Interferon.*

	pH								
Interferon	2	3	4	5	6	7	8	9	Control
Soluble	3.2	3	2	0	0	0	ND[a]	ND	5
Sepharose bound	ND	5.8	4.6	3.8	3.5	1.7	ND	ND	6.5
Sepharose bound	ND	ND	ND	3.7	ND	2.3	2	0	4.1

[a] ND= Not done.

* Autoclaving at pH 2 decomposed the sepharose beads, thus, the preparation could not be assayed for antiviral activity. The results are expressed as the ratio of VSV yield in control/treated cells in log 10.

vation of the antiviral state, interferon itself does not participate in the cellular alteration it induces (5). However, the possibility that soluble interferon indeed might penetrate into the cell cannot be excluded. Cell penetration might be an obligatory step in the inactivation and degradation of the interferon molecule. The latter mechanism could perhaps explain the disproportion frequently observed in the amounts of interferon needed in tissue culture assays when extrapolated to the whole animal.

As expected, interferon bound to Sepharose has altered physicochemical properties. The resistance to thermal inactivation is increased: interferon–Sepharose beads can be autoclaved at low pH without significant loss of antiviral activity. Surprisingly, soluble interferon preparations are also partially resistant when autoclaved between pH 2 and pH 4. Such an increased resistance of interferon to heat (although at lower temperatures, *i.e.*, 56°) at acid pH levels for soluble interferon has already been reported by Marshall *et al.* (6). However, the Sepharose-bound interferon is almost completely resistant to autoclaving in the above-mentioned conditions. Partial resistance of Sepharose-bound interferon to trypsin might be due to protection of some lysyl peptide linkages from proteolysis due to the involvement of ε-amino groups in the iminoester linkage to Sepharose.

Further studies of interferon covalently attached to various carrier molecules are obviously of theoretical and practical significance. As already stated (1), surprising similarities between the mode of action of hormones (such as insulin) and interferon can be drawn. Interferon, just like insulin (7), is active when covalently attached to an insoluble support. The action of both insulin and interferon is inhibited by ouabain, which blocks the membrane-bound ATPase responsible for the uptake of potassium and sodium (8). It can also be postulated that when attached to a suitable support, such as dextran, the interferon molecule could be protected from destruction *in vivo*. Thus, the biological effect of interferon could be prolonged. This problem is, at present, under investigation.

Summary. Properties of Sepharose-bound mouse interferon are compared to those of "native" interferon. The time dependence of the induction of the antiviral state by both preparations is similar, and the dose-response curves obtained with Sepharose-bound interferon are sigmoidal, as those described for native interfe-

TABLE IV. Effect of Pepsin and Trypsin on Sepharose-Bound and Soluble Interferon.*

	Interferon Sepharose-Bound			Interferon soluble	
Pepsin	0.4	ND[a]	ND	ND	ND
Trypsin	ND	1	0.5	0	0
Control	3.2	2.9	3.2	3.2	4.2

[a] ND= Not done.

* The results are expressed as the ratio of VSV yield in control/treated cells in log 10.

ron. Soluble interferon does not diffuse from the interferon–Sepharose particles through the medium to cells not in contact with the beads. No loss of antiviral potency to the medium after repeated cell-to-cell transfers of these particles is observed.

Experiments employing a relatively small ratio between interferon–Sepharose beads and cells indicate that multiple contacts of cells and beads result in increased antiviral activity. Sepharose-bound interferon is more thermostable than native interferon and almost completely resists autoclaving at 110° at pH 3. Likewise, degradation by trypsin is somewhat retarded. Our data indicate that the Sepharose-bound interferon is responsible for the effects observed and that covalent binding of interferon to an insoluble support increases its stability.

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