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Antiviral Effect of Interferon Covalently Bound to Sepharose

(vesicular stomatitis virus/encephalomyocarditis virus/activated Sepharose)

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ABSTRACT **Interferon, covalently bound to Sepharose 4B activated by cyanogen bromide, induces the antiviral state in sensitive cells. The antiviral effect is neutralized by antiserum specific to interferon and is recovered thereafter when the antibody is detached from the interferon by treatment at low pH. Binding interferon to Sepharose increases the stability of the molecule. It is likely that the interferon molecule acts on the cell receptor without being detached from the beads. However, the data do not exclude the possibility of a small loss of interferon, or fragments of it, after contact with the cell.**

The mechanism of interferon uptake by cells and activation of the antiviral state are poorly understood. The existence of interferon-specific receptor sites on cell membranes is generally accepted (1, 2). In addition, we have previously postulated that establishment of the antiviral state is a cooperative phenomenon with the possible involvement of a receptor and an activator site (2). It is not known whether the whole interferon molecule or only fragments of it penetrate into the cell. Another possibility could be that, like insulin (3) or epinephrine (4), interferon does not penetrate into the cell but acts on surface receptors, thus activating a series of events that finally result in the antiviral state. To investigate these possibilities, we covalently attached mouse interferon to Sepharose beads by Porath's technique (5) and assayed the antiviral effect of such an insoluble interferon adduct in suitable cell systems.

MATERIALS AND METHODS

Cell Lines. Mouse L cells and monkey BSC-1 cells were routinely propagated in this laboratory.

Medium. All 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 (EMC) virus were routinely passaged in our laboratory in L cells. Virus was titrated by routine plaque assays in suitable cell systems. EMC was also assayed by means of human erythrocyte agglutination at 4°.

Preparation and Assay of Interferon. Mouse interferon was prepared and concentrated from MSV IF$^+$ cells as described (6). The procedure used for binding of mouse interferon to Sepharose was essentially that of Porath *et al.* (5). Commercial CNBr-activated Sepharose 4B (5 g) (Pharmacia Fine Chemicals AB, Upsala, Sweden) was suspended in 500 ml of 1 mM HCl. The beads were allowed to settle and were washed with an equal volume of 1 mM 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 reference 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 was 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. The final Sepharose preparation was suspended in an equal volume of Eagle's medium.

Control preparations of inactive Sepharose were prepared by first incubating the CNBr-activated Sepharose with ethanolamine, and then with interferon. The procedure was exactly the same as that described above, but the steps concerning the incubation with interferon and ethanolamine were reversed. The protein concentration in the supernatant after the second incubation was 0.57 mg/0.1 ml and it contained 2×10^4 units of interferon per 0.1 ml, indicating that no measurable binding of protein or of interferon to Sepharose had taken place.

Interferon was assayed by incubation of serial 2-fold dilutions of soluble interferon with L cells for 24 hr at 37° [with either microplaques (Falcon) or plastic petri dishes (Falcon 30-mm diameter)]. Sepharose-bound interferon was suspended at a concentration of 12×10^6 beads per ml. Suitable dilutions were prepared therefrom by dilution of aliquots with Eagle's medium. The number of beads per ml was determined by direct count in a hemocytometer. Control beads were similarly incubated at 37° for 24 hr. Then interferon or the beads were removed. The cells were washed and challenged with

Abbreviations: VSV, vesicular stomatitis virus; EMC, encephalomyocarditis; PFU, plaque-forming unit.

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TABLE 1. *Antiviral activity of mouse interferon before and after incubation with CNBr-Sepharose*

Interferon preparation	Antiviral titer (reference units per 0.1 ml)	Protein (mg/0.1 ml)
Starting material	2×10^4	0.6
Supernatant after incubation with CNBr-Sepharose	2×10^2	0.33
Supernatant after Sepharose inactivated by ethanolamine	2×10^4	0.57

The antiviral effect of interferon was expressed in units adjusted to N.I.H. mouse reference interferon.

EMC virus or VSV at a multiplicity of infection = 1 plaque-forming unit (PFU)/cell. After 1 hr, the unadsorbed virus was removed and the cells were incubated with fresh medium for 16 hr. Viral yield was established by routine hemagglutination and plaque titration.

Antiserum to Mouse Interferon was prepared by immunization of goats and was a generous gift of Dr. Fauconnier (7). At a 1:4 dilution, this antiserum completely neutralized the antiviral effect of 800 reference units of soluble mouse interferon.

Protein Content of the different preparations was determined according to Lowry *et al.* (8), with bovine-serum albumin as a reference.

RESULTS

Binding of Interferon to Sepharose. When a preparation of mouse interferon was incubated with Sepharose 4B activated with cyanogen bromide (CNBr-Sepharose), about 99% of the antiviral activity, but only about 50% of the total amount of protein, disappeared from the solution. In control preparations in which interferon was incubated with CNBr-Sepharose treated with ethanolamine, no binding of either interferon or protein occurred (Table 1). CNBr-Sepharose treated with interferon (interferon–Sepharose) or control beads were incubated at different concentrations with sensitive cells. As shown in Fig. 1, interferon activity was only associated with washed interferon–Sepharose particles; CNBr-Sepharose beads inactivated with ethanolamine before incubation with interferon had no antiviral effect. The data indicate that interferon binds covalently through the iminoester sites on the Sepharose, since the binding of interferon was abolished by saturating these sites with ethanolamine.

Neutralization of the Antiviral Activity of Interferon–Sepharose by Specific Antiserum to Mouse Interferon. The antiviral effect of Sepharose-bound, as well as that of soluble, interferon was completely neutralized by interferon-specific antiserum. Subsequent treatment of the Sepharose-bound antigen–antibody complex at pH 2.25 for 30 min at 20°, with 1 M propionic acid, almost completely restored the antiviral effect of the particles (Table 2).

Action of Interferon–Sepharose on Heterologous Cells. Binding of interferon to Sepharose did not alter its known cell species specificity (Table 3). Interferon–Sepharose did not induce the antiviral state in a continuous line of monkey cells (BSC-1), while it was effective in homologous mouse L cells.

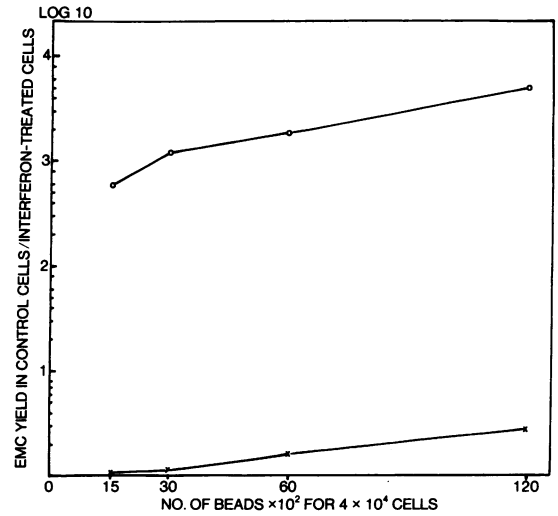

FIG. 1. Correlation between the antiviral activity (expressed as the ratio of EMC yield in control cells per interferon-treated cells) of Sepharose-bound interferon and the number of beads per 4×10^4 cells used. ○, Interferon bound to Sepharose; ×, interferon bound to Sepharose previously saturated by ethanolamine.

Binding of Interferon–Sepharose to Cell Receptors. Friedman (1) has shown that cells treated with interferon at 4° for 4 hr will develop the antiviral state after removal of interferon and subsequent incubation of the cells at 37°. No antiviral effect was observed when the cells were challenged with the virus immediately after removal of interferon. These data suggest that some interferon binds to the cells at 4° in such a

TABLE 2. *Neutralization of antiviral effect of interferon–Sepharose by antiserum to interferon and its recovery at pH 2.25*

	Soluble interferon		Interferon–Sepharose	
	HA	PFU/0.5 ml	HA	PFU/0.5 ml
Control	<2	2.8×10^5	≤2	4.9×10^5
+ Antiserum	2048	2.1×10^8	2048	2.2×10^8
+ Antiserum Treated at pH 2.25	ND	ND	4*	2.1×10^6
Treated at pH 2.25	ND	ND	4*	3.5×10^6
Control EMC	4096	2.8×10^8		
+ Interferon antiserum	2048	1.7×10^8		

The amount of antibody used is equivalent to that required to neutralize 800 units of soluble interferon. EMC virus titers are expressed in hemagglutinating (*HA*) and plaque-forming (*PFU*) units.

* The slight decrease of the antiviral activity is most likely due to a 20% loss of the number of beads during the manipulations.

ND = not done.

TABLE 3. *Cell species specificity of the antiviral effect of interferon bound to Sepharose*

	VSV titer (PFU/0.5 ml)	
	Monkey BSC-1 cells	Mouse L cells
Interferon–Sepharose	10^6	$<10^2$
Soluble interferon	1.2×10^6	$<10^2$
Control virus	8×10^5	2.2×10^5

Antiviral activity of soluble and Sepharose-bound mouse interferon in L cells and monkey BSC-1 cells challenged with VSV.

manner that it cannot be removed by washing, but that cellular metabolism at 37° is required to establish the antiviral state. When similarly tested, Sepharose-bound interferon, in contrast to free interferon, was completely removed from the cells. The data in Table 4 indicate that, under these conditions, free interferon protects the cells against EMC, whereas Sepharose-bound interferon is inactive. These data provide strong evidence that Sepharose-bound interferon is not liberated upon cell contact at 4°.

Separation of Interferon–Sepharose and Cells by Membranes. We attempted to demonstrate the necessity of contact between the cells and Sepharose-bound interferon for antiviral activity. In such experiments, porous membranes (Vyon Filter LKB, 4990-61, 28-mm diameter) were placed between cells and beads. In control sets, interferon was inoculated on top of the filters in such a manner that soluble material had to pass through to interact with the cells. The presence of filters abolished the antiviral effect of insoluble interferon, but the effect of free interferon (although reduced) was measurable (Table 5). This loss of activity in free interferon was probably due to adsorption to the filters (Table 5). Millipore filters also prevented the effect of bound interferon, but retained even more free interferon (unpublished).

Repeated Cell-to-Cell Transfer of Interferon–Sepharose. If Sepharose-bound interferon or an active peptide is detached from the beads in contact with the cell membrane, then repeated incubation of the same beads with different cell preparations should lead to a progressive loss of the antiviral effect of the beads.

To investigate this possibility, we incubated 1.1×10^4 beads with about 10^6 cells grown in Falcon plastic petri dishes.

TABLE 4. *Lack of establishment of the antiviral state by interferon–Sepharose previously incubated with the cells at 4°*

	Preincubated 5 hr at 4°		Preincubated 5 hr at 37°	
	HA	PFU/0.5 ml	HA	PFU/0.5 ml
Interferon–Sepharose	>2048	6.8×10^8	512	1.6×10^8
Interferon	256 512	7.7×10^7	256	6.2×10^7
Control EMC	>4096	5×10^8	>4096	6.5×10^8

L cells were incubated at 4° or 37° for 4 hr. Then they were washed three times, replenished with fresh medium, and incubated for a further 18 hr at 37°. Then they were challenged with EMC, multiplicity of infection = 1.

TABLE 5. *Separation of Sepharose-bound interferon from the cells by filters*

	EMC (PFU/0.5 ml)	
	With filter	Without filter
Interferon–Sepharose	3.9×10^8	4.8×10^5
Soluble interferon	5.2×10^7	1.7×10^5
Control EMC	3.5×10^8	3×10^8

Antiviral effect of insoluble and soluble interferon separated from the cells by a filter. The small loss of soluble interferon activity is probably due to unspecific adsorption to the filter.

Beads and cells were kept in contact for 4 hr at 37° and were gently shaken every 2 hr. Then the beads were removed, centrifuged, counted and incubated for another 4 hr under the same conditions with the same number of fresh cells. This procedure was repeated three times. After the last transfer, the beads were incubated with 10^6 cells for 18 hr. Then they were removed, and the cells were challenged with VSV or EMC virus. Control interferon–Sepharose beads were serially passaged at 37° but without cells. Another set of control preparations, maintained during the same period at 4°, was also tested with the cells. Repeated contact of interferon–Sepharose beads with four different sets of cells did not significantly decrease the antiviral effect of the beads (Table 6).

Thermal Stability of Sepharose-Bound Interferon at 56°. Soluble and insoluble interferon were incubated for 1 hr at 56° and then assayed. Heating at 56° completely inactivated soluble interferon, while the Sepharose-bound interferon was only slightly affected (Table 7).

DISCUSSION

Mouse interferon, like many other proteins, can be covalently attached to activated CNBr-Sepharose. No such attachment occurs when the CNBr-Sepharose is previously saturated with ethanolamine, lending strong support to our conclusion of covalent linkages between interferon and Sepharose. In our experiments, almost 100% of interferon activity was bound, despite the fact that most of the total protein content of the interferon solution used consisted of contaminants. This result seems to indicate that preferential binding of interferon occurred, since only about 50% of the total amount of protein originally present was attached to the Sepharose particles. The major part of the bound interferon appeared to be active, indicating that the linkage(s) to Sepharose does

TABLE 6. *Antiviral activity of interferon–Sepharose before and after four cell-to-cell transfers*

	EMC (PFU/0.5 ml)	VSV (PFU/0.5 ml)
Interferon–Sepharose control 4°	1.5×10^7	7.4×10^3
Interferon–Sepharose control 37°	3.7×10^7	1.2×10^4
Interferon–Sepharose at the fourth cell-to-cell transfer	4×10^7	2×10^4
Control virus	5.6×10^8	1.2×10^7

TABLE 7. *Heat treatment of interferon–Sepharose*

	EMC (PFU/0.5 ml)
Interferon–Sepharose control	2×10^7
Interferon–Sepharose 56°	4.6×10^7
Interferon control	6×10^7
Interferon 56°	4.6×10^8
Control EMC	7.4×10^8

not alter the active site of the interferon molecule. Since cellular species specificity is also retained, binding to Sepharose does not block the molecular site responsible for this function. An additional argument for covalent binding of interferon and Sepharose is the neutralization of the antiviral effect of insoluble interferon by antiserum specific to interferon, followed by recovery of almost full biological activity after removal of the antibody by treatment at low pH.

The insoluble interferon molecule is more heat stable, since, in contrast to soluble interferon, activity is retained after heating at 56° for 1 hr. This increased stability could be of practical value for more reliable interferon standards, or for therapeutic applications.

It has been proposed that induction of the antiviral state by interferon is a cooperative event involving a receptor and activator site (2). For activation of the antiviral state the interferon molecule, could (*i*) be taken up by the cell; (*ii*) be cleaved, resulting in liberation of an active residue that then penetrates into the cell; or (*iii*) interact with the cell membrane without penetration. Our data are in favor of this hypothesis, since: (*i*) repeated cell-to-cell transfer of Sepharose-bound interferon did not significantly decrease its antiviral properties; (*ii*) physical separation of cells and beads abolished it; and (*iii*) contact with cells at 4° did not result in significant induction of the antiviral state after removal of the beads and transfer to 37°. It is impossible, however, to exclude that a few interferon molecules or fragments therefrom might be detached from beads in contact with the cell membrane. If so, the amount liberated must be so small that its discovery requires more sensitive methods than those presently available.

It is interesting to compare some of the properties of interferon and insulin. Both are proteins of low molecular weight, diffusing in the tissue and in the circulation, and their biological effects are inhibited by ouabain (9, 10). In addition, both are active when bound to Sepharose (3), suggesting that for their biological activity no penetration is required. This analogous behavior of interferon and insulin represents an interesting lead for future investigations of interferon and of hormones.

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