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Electrophoretically pure mouse interferon exerts multiple biologic effects

(antitumor effects/cell multiplication/histocompatibility antigens/immune response/delayed type hypersensitivity)

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ABSTRACT **Electrophoretically pure mouse interferon was examined for a number of biologic effects previously ascribed to crude or partially purified interferon preparations. These effects include: inhibition of the growth of a transplantable tumor in mice; inhibition of cell multiplication of mouse tumor cells *in vitro*; enhancement of the expression of histocompatibility antigens on mouse tumor cells *in vitro*; inhibition of antibody formation *in vitro*; inhibition of sensitization to sheep erythrocytes and the expression of delayed type hypersensitivity in mice; enhancement of natural killer cell activity *in vivo* and *in vitro*; enhancement of cell sensitivity to the toxicity of poly(I)·poly(C); and enhanced production ("priming") of interferon production *in vitro*. Our results establish that the molecules responsible for the antiviral action of interferon are also responsible for these varied biologic effects.**

In many animal cells, viral infection results in the production of potent antiviral glycoproteins called interferon (1). In addition to interferon's well-known antiviral action, preparations of it have been reported to exert multiple effects on cells [i.e., inhibition of the multiplication of normal and tumor cells in culture (2–5), inhibition of the growth of a variety of transplantable tumors in mice (6–8), modulation of the synthesis and release of specific cellular products (9–16), modification of the cell surface (17–21), and modulation of both the humoral (22–27) and cell-mediated immune responses (28–34)]. Although experimental results support the hypothesis that the antiviral action of interferon is only one manifestation of its effect on cells (35–37), it is important to emphasize that these experiments were undertaken using preparations in which interferon protein constituted at most only 1% of the total protein content. We have recently succeeded in preparing electrophoretically pure (EP) mouse interferon (38), and we present herein results which show that a number of the biologic effects previously observed with crude or partially purified preparations are also observed with this EP interferon. These results provide the most direct evidence to date that interferon not only inhibits viral multiplication but also exerts a variety of important biologic effects on cells.

MATERIALS AND METHODS

Partially purified mouse interferon

Mouse C-243 cell interferon was prepared, assayed, partially purified, and concentrated as described (39). The specific activity of the individual preparations ranged from 1×10^7 to 2.9×10^7 mouse interferon reference units/mg of protein.

Electrophoretically pure (EP) mouse interferon

Mouse C-243 cell interferon was prepared as above but was kept for at least 2 weeks at pH 2 before purification. (It was not concentrated by precipitation with ammonium sulfate.) Purification was achieved by two-step affinity chromatography, first on a poly(U)-agarose column (40) and then on a column of sheep anti-mouse interferon globulin coupled to Affigel (38, 41).

We may summarize the criteria of purity of the EP interferon preparation after sequential affinity chromatography as follows: After concentration, the purity of the eluted material was assessed by electrophoresis on 15% polyacrylamide slab gels in the presence of sodium dodecyl sulfate (38). When interferon was electrophoresed at high concentrations (i.e., at least 6×10^6 mouse interferon reference units per slot in a volume of 20 or 30 μl) only two protein bands, migrating as if their molecular weights were 35,000 and 22,000, were observed. When slices of the unstained gel corresponding to these two bands were eluted and tested for biological activity, there was an excellent correlation between antiviral activity and the stained bands. This preparation can be considered electrophoretically pure because no other bands were detected (the limit of detection with the method in our hands was 0.07 μg of either bovine serum albumin or ovalbumin). In one experiment we put 2.5 times as much material on the gel by starting the electrophoresis with 20 μl and reloading twice, each time with 15 μl (using a longer stacking gel). The two interferon bands were more diffuse than those obtained under normal conditions (which was to be expected in view of the way the material was placed on the gel), but again no other bands were observed. Electrophoretic analysis of three different interferon preparations revealed only these two bands in the absence of any other stainable bands. In addition to electrophoresis in a 15% gel, the pure material was run in a 13% gel and in a 10–20% gradient gel, and each time antiviral activity was present in gel slices corresponding to the two stained bands.

In all experiments described in *Results*, "EP interferon" refers to preparations containing both proteins. Where indicated, electrophoretically separated fractions were used.

Further evidence for the purity of the material was obtained by changing the method for staining after electrophoresis. When the gels were stained for carbohydrate with periodic acid/Schiff reagent, instead of Coomassie brilliant blue, again only the two interferon bands were seen. The M_r 35,000 band

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Abbreviations: EP, electrophoretically pure; i.p., intraperitoneally; EA, Ehrlich ascites; poly(I·C), poly(riboinosinic acid)·poly(ribocytidylic acid).

stained almost as intensely with Schiff reagent as with Coomassie brilliant blue, whereas the M_r 22,000 band stained only faintly (38). Changing the electrophoresis buffer from Tris/glycine (42) to 0.01 M sodium phosphate buffer again revealed only two protein bands after staining, comigrating with the antiviral activity. In this case, the high molecular weight form migrated as if its molecular weight were 38,000 rather than 35,000, confirming the results of Stewart *et al.* (43). The difference in migration rates in different buffers obtained with the higher molecular weight component can be attributed to its greater carbohydrate content (as compared to the 22,000-dalton component, whose migration is identical in Tris/glycine and phosphate buffers).

The specific activities of three different EP interferon preparations used in these studies were 9.9×10^8, 1.2×10^9, and 1.5×10^9 reference units/mg of protein as determined by the method of Böehlen *et al.* (44). [These values are in accord with our previously published value of 2.4×10^9 reference units/mg protein (38).]

To prevent loss of activity of highly purified interferon, bovine serum albumin, 100 μg/ml in phosphate-buffered saline, was added to the EP interferon. For purposes of comparison it was at times also added to the partially purified interferon.

The partially purified and EP mouse interferon preparations exhibited antiviral activity on mouse L cells challenged with vesicular stomatitis virus but did not exhibit antiviral activity on human T98G neuroblastoma or chicken embryo fibroblasts.

RESULTS

Antitumor activity (6, 7)

The antitumor activity of mouse interferon was determined in BALB/c mice injected intraperitoneally (i.p.) with Ehrlich ascites (EA) cells (7). Daily injection of both partially purified and EP preparations of mouse interferon resulted in an inhibition of the multiplication of EA cells in the peritoneal cavity (Fig. 1) and an increase in mouse survival time (Table 1).

Inhibition of cell multiplication (2–5)

Sublines of mouse leukemia L 1210 cells sensitive (S) and resistant (R) to interferon (5) were cultivated in the presence of partially purified and EP mouse interferon. In this experiment we eluted the interferon from 2-mm gel slices after electrophoresis of EP interferon in a sodium dodecyl sulfate/polyacrylamide gel. Although the titers were low, EP interferon (fractions 5 and 6) inhibited the multiplication of L 1210_S but not L 1210_R cells (Fig. 2). (The titer of the M_r 22,000 fraction was too low to permit interpretation.)

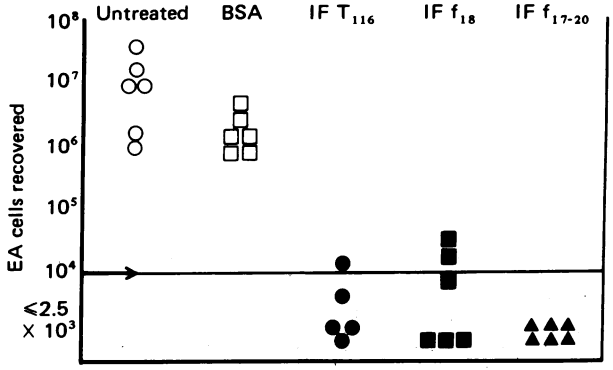

FIG. 1. Effect of mouse interferon preparations on the multiplication of EA cells in the peritoneal cavity. Six-week-old male BALB/c mice were injected i.p. with 10^4 EA cells (horizontal arrow). Five hours later, and daily for 7 days thereafter, mice were injected i.p. with 0.25 ml of bovine serum albumin (BSA) (100 μg/ml), 0.25 ml of partially purified interferon, IF T_{116} (titer 2.56×10^{-5}), or 0.25 ml of two different EP interferons, IF f_{18} or IF f_{17-20} (both having titers of 1.28×10^{-5}). Bovine serum albumin (100 μg/ml) was added to all preparations. After 7 days, the total number of EA cells in the peritoneal cavity of each mouse was determined by using techniques previously described (7). There were six mice per group.

Enhanced expression of H-2 antigens by interferon-treated cells (17)

As shown in Fig. 3, mouse L 1210 cells cultivated in the presence of EP interferon absorbed more mouse alloantiserum than did untreated L 1210 cells or cells treated with bovine serum albumin.

Effects of interferon on the immune system

Inhibition of Antibody Formation *In Vitro* (25–27). C3H mouse spleen cells (6×10^6 cells in 1 ml) were stimulated *in vitro* with sheep erythrocytes (45) and incubated with 0.1 ml of bovine serum albumin (100 μg/ml) or 0.1 ml of partially purified or EP interferon (titer 1.2×10^{-5}). After 5-day incubation, the number of plaque-forming cells was determined. A 94% inhibition in the generation of plaque-forming cells was observed for cultures treated with both interferon preparations in contrast to 11% inhibition for cultures incubated with bovine serum albumin alone.

Inhibition of Delayed Type Hypersensitivity *In Vivo*. The effects of EP interferon on the afferent and efferent pathways of the delayed hypersensitivity reaction were examined. Only the results obtained with the M_r 35,000 fraction eluted from a gel are presented. Comparable results were obtained with the EP interferon prior to electrophoresis.

Table 1. Effect of mouse interferon preparations on survival of mice injected with EA cells

Treatment	No. of mice surviving >160 days/ total no. mice	Harmonic mean survival, days		95% confidence interval
None	0/8	16.7	NS	11.7–29.1
Bovine serum albumin	0/7	18.7	$P \leq 0.01$	12.4–37.9
EP interferon	1/8	34.2	NS	16.7–∞
Partially purified interferon	3/7	51.3		19.6–∞

Six-week-old female BALB/c mice were injected i.p. with 1.6×10^4 EA cells. Two hours later, and daily thereafter for the next 9 days, mice were inoculated i.p. with 0.25 ml of bovine serum albumin (100 μg/ml), partially purified mouse interferon, or EP mouse interferon. The titers of the partially purified and EP interferon preparations were 1.28×10^{-6} and 3.2×10^{-5}, respectively. Bovine serum albumin (100 μg/ml) was added to the EP interferon. NS, not significant.

Sensitization (33). Eight-week-old female BALB/c mice were sensitized intravenously with 10^6 sheep erythrocytes. One group was injected with 8×10^5 units of the M_r 35,000 fraction of EP interferon i.p. prior to sensitization, whereas the control group was injected with phosphate-buffered saline. Four days after sensitization, 10^8 sheep erythrocytes were injected into the left footpad of mice of both groups and footpad swelling was recorded 24 and 48 hr later. Footpad swelling was significantly inhibited in the interferon-treated group (Fig. 4*A*).

Expression (34). This experiment was done under conditions comparable to the foregoing, except that the M_r 35,000 interferon fraction was administered 4½ hr before footpad challenge. Footpad swelling, measured 24 and 48 hr after challenge, was reduced in interferon-treated mice (Fig. 4*B*).

Enhancement of Natural Killer Cell Activity *In Vivo* and *In Vitro* (46–48). Six-week-old male BALB/c mice were inoculated once i.p. with 0.2 ml of bovine serum albumin (100 μg/ml), 0.2 ml of partially purified interferon (titer 9.6×10^{-5}) or EP interferon (titer 6.4×10^{-5}), or they were left untreated. Twenty-four hours later the spleens of the two mice per group were tested individually for cytotoxicity on YAC target cells (46). As can be seen from Table 2, a significant enhancement in cytotoxicity was observed for spleen cells from interferon-treated mice.

Likewise, incubation *in vitro* of splenic lymphocytes for 2½ hours with partially purified or EP interferon (titer 1.2×10^{-4}) enhanced their cytotoxicity for ^{51}Cr-labeled YAC target cells from the baseline value of 29% specific ^{51}Cr release to 55% and 44%, respectively (effector:target cell ratio of 100:1).

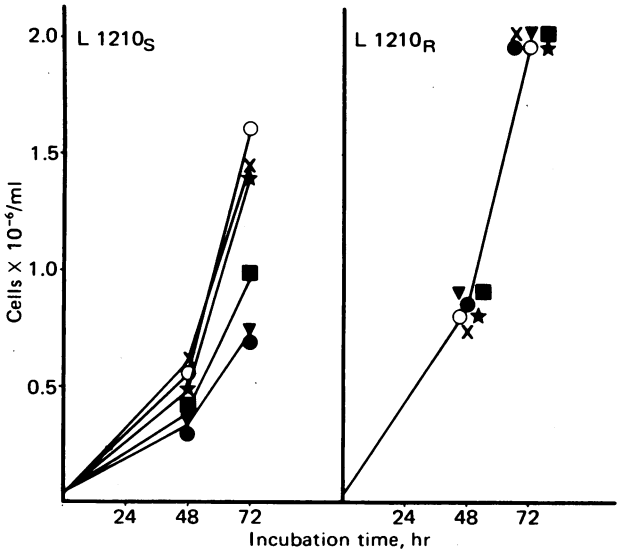

FIG. 2. Effect of mouse interferon preparations on the multiplication of mouse leukemia L 1210_S and L 1210_R cells in culture. L 1210_S and L 1210_R cells were cultivated in suspension culture in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. Cells were seeded at 5×10^4 cells per ml. Cell multiplication was determined after 48- and 72-hr incubation by counting viable cells in a hemocytometer, using the trypan blue dye exclusion test. Each point represents the mean of three different cultures with two cell counts per culture. X, Untreated cultures; ●, partially purified interferon (titer 4×10^{-2}); ▼, eluate fraction 5 of EP interferon (M_r 35,000) (titer 2.4×10^{-2}); ■, eluate fraction 6 of EP interferon (M_r 35,000) (titer 1.2×10^{-2}); ★, eluate fraction 12 of EP interferon (M_r 22,000) (titer 6×10^{-1}); O, eluate fraction 20 (no interferon activity at 4×10^{-1}).

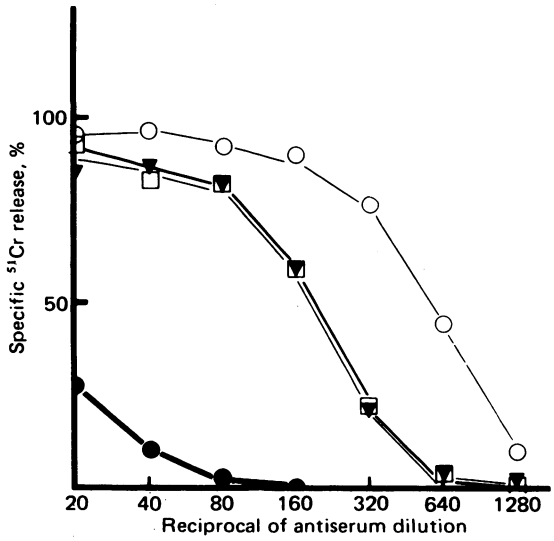

FIG. 3. Effect of EP interferon on the expression of H-2 antigens on the surface of L 1210 cells. Mouse L 1210_S cells were cultivated for 48 hr with bovine serum albumin (100 μg/ml) (▼) or EP interferon (titer 1.6×10^{-3}) and bovine serum albumin (100 μg/ml) (●), or they were left untreated (□). Cells (8×10^6) from each group were incubated for 1 hr at 37°C with a C57BL/6 mouse anti-DBA/2 mouse lymphocyte serum. The cell serum mixtures were centrifuged and the supernatant was titered for cytotoxicity on ^{51}Cr-labeled L 1210 cells as described (17). The activity of the antiserum prior to absorption is also shown (O).

Priming of cells for production of interferon (9, 10)

Mouse embryo fibroblasts were incubated for 18 hr with various concentrations of partially purified and EP interferon prior to addition of 30 μg of poly(I·C) in the presence of DEAE-dextran (100 μg/ml). After 1 hr at 37°C, the inducer was removed, the cell monolayer was washed, and medium was replaced; medium was harvested 24 hr later for determination of interferon content. Pretreatment of cells with 18 or more units of partially purified or EP interferon resulted in an increase in the mean interferon titer from 1:960 to 1:7680.

Enhanced cytotoxicity of poly(I·C) for interferon-treated mouse L cells (50, 51)

As can be seen in Table 3, pretreatment of L cells with either partially purified or EP interferon (after elution of interferon

Table 2. Enhancement of natural killer cell activity in the spleens of BALB/c mice injected with partially purified and EP interferon

Group	Mouse	% specific ^{51}Cr release at effector cell:YAC target cell ratio of 100:1	50:1
Untreated	1	12.7	8.5
	2	13.9	16.2
Bovine serum albumin	1	11.4	9.1
	2	8.1	6.4
Partially purified interferon	1	57.1	49.5
	2	52.3	44.0
EP interferon	1	53.0	47.8
	2	50.3	41.7

Cells were tested in a 4-hr ^{51}Cr release assay with ^{51}Cr-labeled YAC-1 (H-2^a) Moloney mouse lymphoma cells as target cells. Data are presented as specific % lysis according to the formula and methods described in ref. 49. There were five determinations per point.

Table 3. Enhanced cytotoxicity of poly(I·C) for L cells pretreated with partially purified and EP interferon preparations

Interferon pretreatment		Poly(I·C)	% toxicity	
Interferon used	Units	(100 μg/ml)	3 hr	22 hr
None	0	No	0, 0, 0	0, 0, 0
	0	Yes	0, 0, 0	0, 0, 0
Partially purified	100	Yes	100, 100, 100	100, 100, 100
	10	Yes	25, 25, 25	0, 0, 0
	1	Yes	0, 0, 0	0, 0, 0
EP				
Fraction 5	60	No	0, 0	0, 0
	60	Yes	100, 100	100, 100
Fraction 6	30	No	0, 0	0, 0
	30	Yes	100, 100	100, 100
Fraction 12	15	No	0, 0	0, 0
	15	Yes	75, 75	50, 50
Fraction 20	<10	No	0, 0	0, 0
	<10	Yes	0, 0	0, 0

Mouse L-929B cells received from W. Stewart II were cultivated with partially purified or EP interferon for 18 hr. The cell monolayer was then washed, and poly(I·C) (Boehringer), at 100 μg/ml in serum-free medium was added for 2 hr at 37°C. The cell monolayer was again washed and nutrient medium containing 5% calf serum was added. Toxicity was determined by two observers at 3 hr and 22 hr and graded on a scale of 0%, 25%, 50%, 75%, or 100% cell destruction of the cell monolayer. There were three cultures per group. Fractions 5 and 6 were eluates from the gels after electrophoresis in sodium dodecyl sulfate/polyacrylamide (corresponds to M_r 35,000). Fraction 12 was an eluate corresponding to M_r 22,000. Fraction 20 was a gel eluate devoid of interferon activity.

from the gel after polyacrylamide gel electrophoresis of EP interferon in sodium dodecyl sulfate) resulted in cytotoxicity of poly(I·C). Poly(I·C) was not toxic for L cells when cells were not pretreated with interferon.

DISCUSSION

Our results show that, in addition to its antiviral activity, EP interferon exerts a number of other biologic effects previously ascribed to crude or partially purified interferon. The definition of purity is clearly relative and depends on the sensitivity of the methods used to measure possible contaminants. In our studies we have used preparations that showed very distinct interferon bands after polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue. Other bands were not visualized. The lower limit of detection of this method in our hands is 0.07 μg of protein per band, with either bovine serum albumin or ovalbumin as reference proteins. Although this material may not be chemically pure, we believe our results show that the molecules responsible for the antiviral effect are also responsible for the biologic effects described herein.

We are obliged therefore to pose the question: how can one substance exert such a variety of effects on cells? It seems likely that interferon triggers a cascade of events. Some effects occur rapidly and may stem from the initial interaction of interferon with the cell surface [i.e., diminished transport of thymidine (52), reduced release of labeled uridine (53), and enhanced excitability of neurons (54)], whereas other effects occur much later and reflect modifications of cell synthetic processes. Thus, recent work on the mode of action of interferon *in vitro* indicates that it influences control mechanisms of protein synthesis, the ultimate effect being determined by the physiologic state of the cell and by several variables such as the local concentrations of double-stranded RNA, ATP, and tRNA (55). It seems possible that interferon may also influence other metabolic processes such as energy utilization. In view of these considerations, it is not surprising that interferon can either inhibit or enhance certain cell functions (9–16, 35–37).

In addition to having theoretical interest, our results are also pertinent to the clinical usefulness of interferon. Relatively crude and partially purified human leukocyte interferon preparations are currently being used in the treatment of patients with different neoplasms, and some of the preliminary results are most encouraging (56–60). The results presented

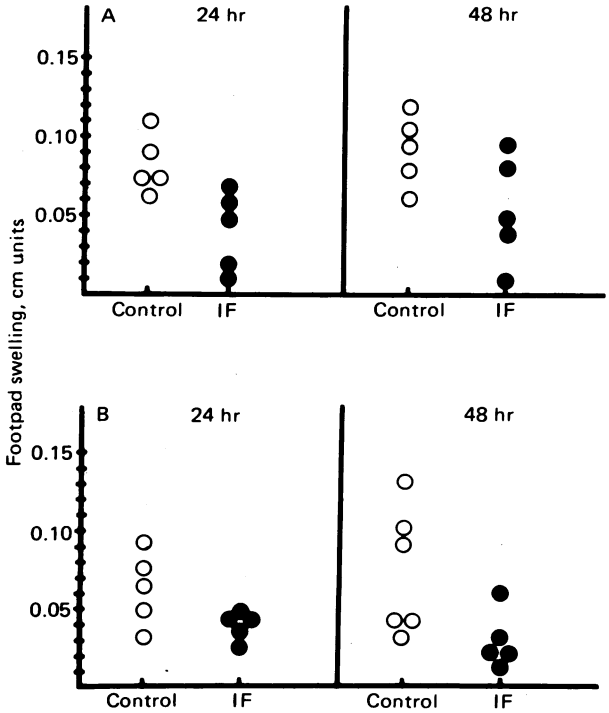

FIG. 4. Effect of the M_r 35,000 fraction of EP interferon on the sensitization (*A*) and the expression (*B*) of delayed hypersensitivity to sheep erythrocytes. A cm unit is defined in refs. 33 and 34. ○, Control mice sensitized and challenged without interferon treatment; ●, mice sensitized and challenged but treated with interferon (IF), either before sensitization (*A*) or before footpad challenge (*B*).

above show that, in one mouse transplantable tumor system, interferon itself is responsible for an antitumor effect. Likewise, because interferon interacts with the immune system and affects the division and function of normal cells, therapeutic uses may now be envisaged for interferon that have not been heretofore considered.

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