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Mouse fibroblast (type I) and immune (type II) interferons: Pronounced differences in affinity for gangliosides and in antiviral and antigrowth effects on mouse leukemia L-1210R cells

(interferon action/carbohydrate specificity/interferon-resistant cells)

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ABSTRACT **Different interferons can be obtained from the same animal species depending on the cells and (or) the inducers used. Interferons of type I and type II differ not only antigenically but also in molecular weight and stability at low pH. We have investigated whether mouse type I and type II interferons also differ in properties relating to their biological action. We present evidence which suggests that the molecular mechanism leading to antiviral and antigrowth effects induced by both types of interferon in susceptible cells must differ in at least one important step. Antiviral and antigrowth activities of type I but not of type II interferon are both inhibited when gangliosides are added to cell cultures together with the interferon. Whereas type I interferon strongly binds to ganglioside affinity columns and can be eluted with solutions of *N*-acetylneuraminyllactose, type II interferon passes through such columns unretarded. L-1210 mouse leukemia cells (L-1210S) respond equally well to antiviral and antigrowth activities of type I and type II interferons. Type I interferon-resistant L-1210 cells (L-1210R), derived from L-1210S cells after continuous culture in the presence of mouse fibroblast interferon, lack antiviral and antigrowth response to mouse type I interferon [Gresser, I., Bandu, H. T. & Brouty-Boyé, D. (1974) *J. Natl. Cancer Inst.* 52, 553–559]. However, these cells display the same sensitivity toward type II interferon as do the parent L-1210S cells from which they were derived and respond equally well to its antiviral and antigrowth activities.**

Interferons are a group of proteins or glycoproteins produced by different vertebrate cells in response to various inducers such as virus, double-stranded RNA, lectins, endotoxins, and other unrelated structures. Increasing evidence suggests that interferons not only confer protection against virus but also inhibit cellular growth and influence a number of processes involved in the immune response (1–3). The type of interferon produced in response to virus or polyinosinicpolycytidylic acid is generally called type I interferon, whereas that produced by lymphocytes in response to specific antigens or mitogens is referred to as type II interferon or immune interferon (2, 4, 5). Type I and type II interferons are distinctly different in regard to molecular weight, antigenic specificities, and stability at low pH (2, 4, 5). Mouse fibroblast (type I) interferon consists of two antigenically similar polypeptides of apparent molecular weights 22,000 and 35,000 (6). Mouse type II interferon, produced in spleen cells of mice sensitized with bacillus Calmette–Guérin after challenge with old tuberculin or with staphylococcus enterotoxin A as the inducer, reveals two components upon gel filtration with apparent molecular weights of approximately 40,000 and 70,000–90,000 (7, 8). Antibody against mouse type I interferon does not neutralize mouse type II interferon, indicating different antigenic properties of the interferons (4). Treatment at pH 2 for several hours has no effect on biological activities of mouse type I interferon but inactivates type II interferon completely (4, 7). Despite these differences in cellular origin, mode of induction, and molecular properties, it appears that both types of interferon are able to induce both cell and virus inhibitory activities in the same target cells equally well.

Mouse fibroblast interferon interacts specifically with carbohydrate-containing cell membrane constituents. Its antiviral action is inhibited after preincubation of target L cells with certain plant lectins, such as the lectin from *Phaeseolus vulgaris* (9) or the nontoxic agglutinin from *Abrus precatorius* (unpublished observation) and after preincubation of the interferon itself with gangliosides (10, 11). This type of interferon binds to carbohydrate constituents of the ganglioside molecules and can be eluted from affinity columns containing covalently bound gangliosides by solutions of *N*-acetylneuraminyllactose, the trisaccharide common to most gangliosides (12). Furthermore, pretreatment of mouse SV/ALN cells with exogenous gangliosides under conditions that lead to incorporation into the membranes of these cells increases their sensitivity to the antiviral action of mouse fibroblast interferon several-fold, suggesting that interaction of this type of interferon with cell membrane gangliosides is of functional significance (13).

It is conceivable that structurally distinct interferons such as type I and type II also differ in their mechanisms of action. We have investigated this question by comparing the effects of gangliosides on antiviral and antigrowth effects of mouse type I and type II interferons. We have also compared antiviral and antigrowth activities of both interferons on a subline of mouse leukemia cells resistant to fibroblast interferon (14).

MATERIALS AND METHODS

Cells. Fibroblast interferon-sensitive and -resistant mouse leukemia L-1210 cells (L-1210S and L-1210R, respectively) were kindly provided by Ion Gresser and were cultured in RPMI-1640 medium (GIBCO) supplemented with 5% fetal bovine serum. L_{929} cells were routinely propagated in Eagle's minimal essential medium plus Hanks' salts (GIBCO) containing 10% fetal bovine serum.

Reagents. Partially purified mouse fibroblast (type I) interferon was supplied by Kurt Paucker and had a specific ac-

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Abbreviations: IU, National Institutes of Health reference units; EMV, encephalomyocarditis virus; VSV, vesicular stomatitis virus.

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tivity of 2.4×10^7 National Institutes of Health reference units/mg (IU/mg). Mouse immune (type II) interferon was produced in spleen cells, from *nude* heterozygous mice (*nu*/+) or BALB/C mice (15), stimulated with lectin from *P. vulgaris*. Spleen cells (10^7 cells/ml) were suspended in RPMI-1640 medium supplemented with 5% fetal bovine serum, 2 mM glutamine, and 40 μg of gentamycin per ml. They were incubated with 3 μg of purified lectin (Wellcome Laboratories) per ml in 90-mm petri dishes for 24 hr at 37°C. Cells were removed by centrifugation and the supernatant was stored at −70°C until used. Antiviral titers were between 200 and 500 IU/ml in different batches. Crude type II interferon preparations were fractioned with ammonium sulfate at 4°C. The precipitate obtained at 42% saturation with ammonium sulfate was removed by centrifugation and the supernatant was concentrated to 10–20% of its original volume by vacuum dialysis. The concentrate was further purified by chromatography on a column of blue Sepharose CL-68 (Pharmacia) as described (16). Interferon eluting from this column had a specific activity of $1–2 \times 10^5$ IU/mg and was used in these studies.

N-Acetylneuraminyllactose, mixed bovine brain gangliosides, and polylysine (M_r, 30,000) were from Sigma, CNBr-activated Sepharose was from Pharmacia, and individual gangliosides were from Supelco. Gangliosides G_{M3} and G_{M2} were kindly provided by Subhash Basu. Individual gangliosides are designed according to Svennerholm (17). Their purity was established by thin-layer chromatography (see below). G_{M1}, G_{M2}, and G_{M3} showed single spots after exposure to resorcinol spray or iodine vapor. G_{D1a} was slightly contaminated by a resorcinol-positive component with the mobility of G_{M2}; G_{T1b} showed one additional spot that moved identically to G_{D1b}. Both contaminants were present in amounts of 10% or less as judged from the relative intensities of the spots. Ganglioside affinity columns were prepared as described (10). The procedure involved coupling of polylysine to CNBr-activated Sepharose (18), followed by the attachment of mixed bovine brain gangliosides with carbodiimide (19).

Antiviral Assay. *Assay 1.* This was carried out with L_{929} cells and encephalomyocarditis virus (EMV). Approximately 10^5 cells in 1 ml of minimal essential medium plus 10% fetal bovine serum was seeded into 16-mm wells of MultiWell tissue culture plates (Falcon) and kept at 37°C in a CO_2 incubator. The following day, the medium was removed and cells were incubated with appropriate dilutions of interferon in serum-free medium containing 50 μg of bovine serum albumin per ml (0.5 ml/well). Where indicated, interferon solutions were preincubated with glycolipids at 37°C for 30 min prior to addition to the cell monolayers. Control cells were incubated under identical conditions but in the absence of interferon. After 24 hr at 37°C the medium was removed and the cells were infected with EMV at a multiplicity of infection of 0.1; 0.5 ml of an appropriate virus suspension in medium containing 2% fetal bovine serum was added to each well. After 1 hr at 37°C, the medium and nonadsorbed virus was removed and the cells were incubated in 0.5 ml of fresh medium plus 2% fetal bovine serum for 16–17 hr at 37°C. The cultures were then placed at −80°C for at least 30 min and then thawed, and the virus yield was determined by hemagglutination of serial 1:2 dilutions of the virus suspensions (20). EMV titers are expressed as the reciprocals of the highest dilutions that still showed hemagglutination. The accuracy of this assay is about ±1 dilution. When the effects of type I and type II interferons and those of different gangliosides on these interferons were compared, experiments were performed simultaneously with the same batch of L cells under identical conditions. This was necessary because interferon sensitivity and viral yield were somewhat variable from culture to culture. Concentrations of interferon used in individual experiments refer to appropriate dilutions of the original solution, whose interferon titers were determined by comparing their antiviral activities to those of National Institutes of Health standard G 002-49-511; 1 IU/ml refers to an interferon concentration that results in 50% inhibition of viral yield compared to the standard.

Assay 2. Because EMV does not grow in L-1210 cells, antiviral effects of type I and II interferons on these cells were determined with vesicular stomatitis virus (VSV). L-1210S and L-1210R cells in 0.5 ml of RPMI-1640 medium containing 5% fetal bovine serum were added to 35 mm petri dishes (1.5×10^6 cells per dish), followed by 0.5 ml of appropriate dilutions of interferon type I or type II in the same medium. As controls, cells were simultaneously incubated in dishes with medium without interferon. After 24 hr at 37°C in a CO_2 incubator, all cells were infected with VSV at a multiplicity of infection of 0.1 by adding 1 ml of appropriate virus suspension in RPMI medium plus 5% serum to each dish. After a further 18 hr at 37°C, the viral yield was determined by plaque assay. Viral yield is expressed as % of plaque-forming units with respect to the yield from the control cells. Plaque assays were carried out in triplicates and were reproducible within a factor of 2.

Growth Studies. L-1210R and L-1210S cells were grown without agitation in plastic vials in RPMI medium containing 5% fetal bovine serum at 37°C in a CO_2 incubator; between 0.3 and 1 ml of culture medium was used in different experiments. Cells were counted in a Coulter Counter. When L-1210R and L-1210S cells were compared, cultures were studied simultaneously and under identical conditions.

Column Chromatography. Sepharose beads containing covalently linked gangliosides were placed in a pasteur pipette (0.2 ml packed volume) containing a small amount of glass wool. Columns were washed with 3 ml of minimal essential medium containing 50 μg of bovine serum albumin per ml. Interferon dissolved in medium/albumin (1 ml) was placed on the columns, which were eluted with medium/albumin at a flow rate of no more than 1 drop per min at room temperature. Fractions (1 ml) were collected and, after serial 1:2 dilution interferon titers were determined in each fraction by using assay 1. Columns onto which mouse fibroblast interferon had been loaded were eluted with medium/albumin and then with 0.07 M *N*-acetylneuraminyllactose in medium at pH 2.

Other Analytical Procedures. Thin-layer chromatography was carried out with silica gel plates G60 (Merck); development was with chloroform/methanol/water, 65:45:9 (vol/vol), or *n*-propanol/0.2% aqueous $CaCl_2$, 80:20 (vol/vol). The spots were located with iodine vapor or with resorcinol spray (21). The sialic acid of gangliosides was determined with thiobarbituric acid after hydrolysis in 0.1 M sulfuric acid (22).

RESULTS

Effects of Gangliosides. A mixture of bovine brain gangliosides containing mainly G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b} (23) progressively inhibited the antiviral action of mouse fibroblast interferon when solutions of this interferon were preincubated with the glycolipid solutions prior to addition to target L cells (Fig. 1). Complete reversal of the antiviral effect of fibroblast interferon at 20 IU/ml occurred at a ganglioside concentration corresponding to 20 μM sialic acid. On the other hand, pretreatment of type II interferon with the same ganglioside mixture under identical conditions had no effect on antiviral activity of this interferon, even at concentrations corresponding to 100 μM sialic acid (not shown). In similar experiments using 100 μM solutions of individual gangliosides, no effect on type II interferon was observed, whereas antiviral activity of type

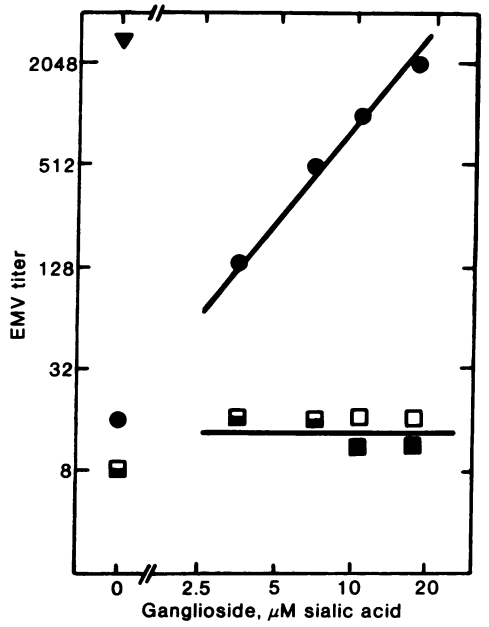

FIG. 1. Effects of bovine brain gangliosides on antiviral activities of mouse type I (●) and type II (□) interferons. Interferon solutions in minimal essential medium (20 IU/ml) were preincubated with the indicated concentrations of gangliosides at 37°C for 30 min prior to addition to the L-cell monolayers. Antiviral assays were by assay 1. ■, Type II interferon after passage through a Sepharose-ganglioside column (see Fig. 2); ▼, EMV titer in the absence of interferon.

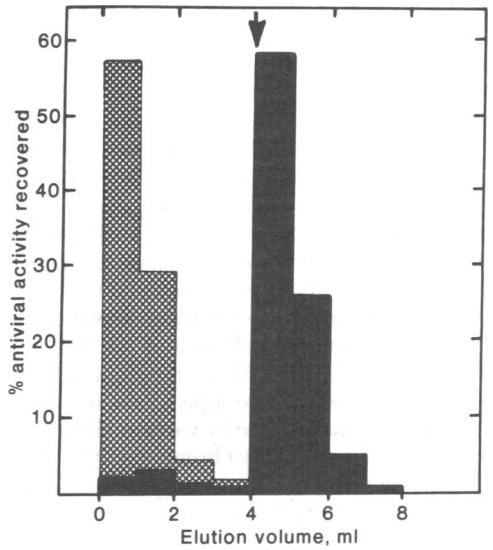

FIG. 2. Elution profiles of mouse type I (solid bars) and type II (hatched bars) interferons after application to Sepharose-ganglioside affinity columns. One milliliter of interferon solution (2×10^3 IU of type I or 10^3 IU of type II) in minimal essential medium plus bovine serum albumin (50 μg/ml) was loaded onto a column containing 0.2 ml of the Sepharose-ganglioside adduct. Columns, run simultaneously under identical conditions, were first eluted with medium/albumin alone. Where indicated by the arrow, elution of the column containing type I interferon was continued with 0.07 M *N*-acetylneuraminyllactose in medium/albumin at pH 2. Columns with type II interferon were not further eluted because the majority of the antiviral activity (91%) passed through the column unretarded and type II interferon is destroyed at pH 2. Antiviral activity in each fraction was determined by assay 1. A small amount of type I interferon passed through the column unretarded (7%); the remainder (89% of that applied) was eluted with *N*-acetylneuraminyllactose.

I interferon was completely inhibited at these or lower concentrations (Table 1). Antiviral activity of a crude type II interferon preparation (approximately 10^3 IU/mg) obtained from cultured spleen cells of mice sensitized with *Mycobacterium bovis*, strain BCG, after induction with old tuberculin [kindly provided by G. Sonnenfeld and T. C. Merrigan (6)] was also found to be insensitive to inhibition by the bovine brain ganglioside mixture used in the above studies (unpublished observation).

Effects of Ganglioside Affinity Columns. More than 90% of the antiviral activity of mouse fibroblast interferon was retained on a column containing covalently bound gangliosides (Fig. 2). The bound activity was eluted with 0.07 M *N*-acetylneuraminyllactose at pH 2 in accordance with previous results (11, 12). On the other hand, the antiviral activity of type II interferon passed through identical columns unretarded.

After passage through the affinity column, type II interferon was still insensitive to ganglioside inhibition, excluding the possibility that a noninterferon contaminant with high affinity for gangliosides was removed by this procedure (Fig. 1). To exclude enzymatic destruction of gangliosides, we preincubated a solution of the bovine brain ganglioside mixture with type II interferon (at an interferon concentration 10 times higher than used in the antiviral assay) for 24 hr at 37°C; then we heat-inactivated the interferon (2 min at 100°C) and assayed the treated gangliosides for their inhibitory action on fibroblast interferon. As a control we used a ganglioside solution preincubated and then heated identically, but in the absence of type II interferon. Although there was a small decrease in inhibitory potency of the ganglioside solutions after this treatment (approximately 30%), there was no significant difference between the solutions preincubated with type II interferon and those preincubated in medium alone. Thus, failure of type II interferon to bind to ganglioside affinity columns and to be inhibited by gangliosides must be due to the interferon molecule itself and not to contaminating factors that either compete by binding to gangliosides or degrade gangliosides to noninhibitory breakdown products.

Table 1. Effects of individual gangliosides on antiviral activities of mouse type I and type II interferons

Ganglioside	Conc. for complete inhibition of type I interferon, μM*	Effect of 100 μM ganglioside on type II interferon*
G_{M3}	100	None
G_{M2}	15	None
G_{M1}	45	None
G_{D1a}	30	None
G_{T1b}	15	None

* Interferon present at 30 IU/ml.

Antiviral and Antigrowth Effects of Type I and Type II Interferons on L-1210S and L-1210R Cells. Whereas both interferons protected L-1210S cells against VSV equally well, there was a difference when L-1210R cells were treated with type I or with type II interferon under identical conditions (Fig. 3). As expected, from the original observations by Gresser *et al.* (14), mouse fibroblast interferon protected these cells against VSV only marginally, even at a concentration of 1000 IU/ml. On the other hand, they responded to the antiviral effect of type II interferon with almost the same sensitivity as did the parent cells from which they were derived. Antigrowth activity of both types of interferon on L-1210S and L-1210R cells is shown in Fig. 4. The presence of comparable concentrations of either of the two interferons during culture for 3 days inhibited growth of L-1210S cells to the same extent, resulting in approximately 50% inhibition at concentrations of 1000 IU/ml of each inter-

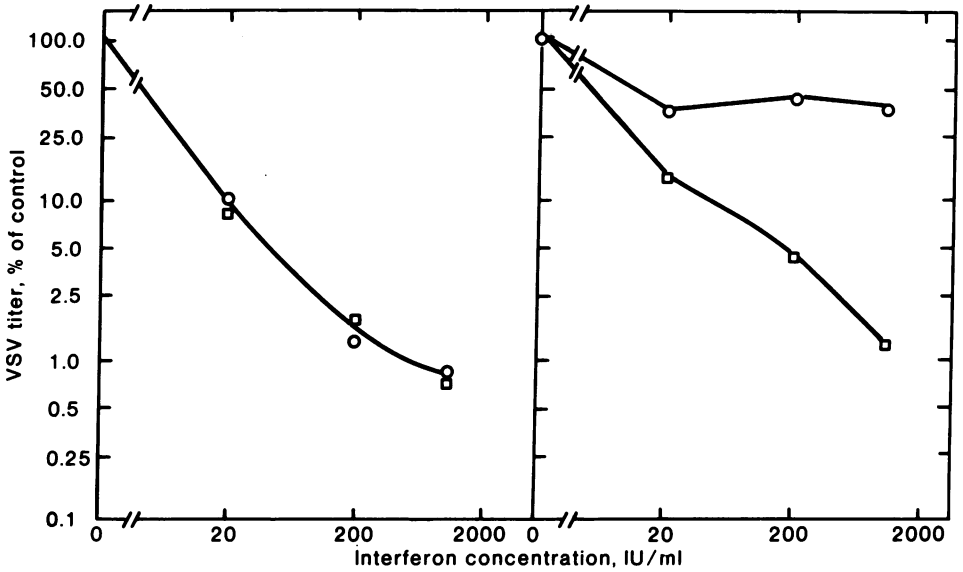

FIG. 3. Comparison of antiviral activities of type I (○) and type II (□) interferons on L-1210S (*Left*) and L-1210R cells (*Right*). Cells were incubated with the indicated concentrations of both interferons and the viral yield after infection with VSV was determined by plaque assay with assay 2. Virus yields in control cells were 12 and 5 × 10^6 plaque-forming units/ml for L-1210S and L-1210R cells, respectively.

feron. As in the antiviral assay, L-1210R cells were highly resistant to the antigrowth activity of type I interferon (14) but showed the same response to the antigrowth activity of type II interferon as did the L-1210S parent cells. Furthermore, the antigrowth effect of type I interferon was completely reversed by the presence of gangliosides, whereas that of type II interferon was not affected.

That resistance of L-1210R cells to fibroblast interferon was not due to gangliosides or other inhibitors specific for the fibroblast variety shed from these cells into the medium was shown by assaying the antigrowth activity of fibroblast interferon on L-1210S cells suspended in 4-day-old culture medium of L-1210R cells under conditions identical to those described in Fig. 4. Control L-1210S cells were suspended in 4-day-old culture medium of L-1210S cells. In comparison to these control cells, the antigrowth effect of fibroblast interferon (1000 IU/ml) on L-1210S cells in L-1210R cell-derived medium was the same, indicating that different responses at L-1210R cells to the different interferons were due to the cells themselves and not to type I interferon-specific inhibitors shed into the medium by L-1210R cells, in accordance with results reported by Gresser *et al.* (14).

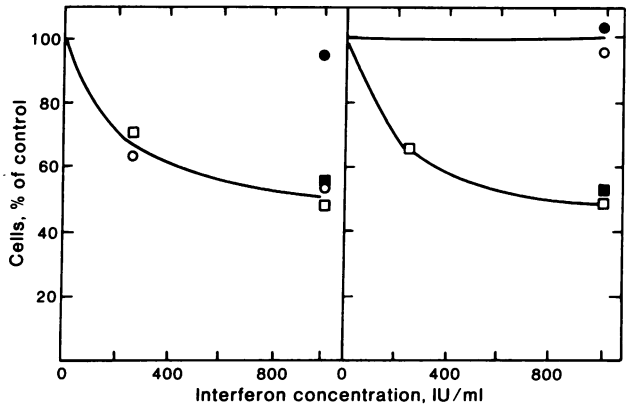

FIG. 4. Antigrowth activities of type I and type II interferons on L-1210S (*Left*) and L-1210R cells (*Right*) and of the effects of bovine brain gangliosides on antigrowth activity. Cells were seeded at an original density of 8 × 10^4 L-1210S and L-1210R cells per ml in 0.3 ml total volume. Cells were counted after 3 days of growth. The cell number in control cultures (100%) was 3.1 × 10^5 L-1210S cells per ml and 2.6 × 10^5 L-1210R cells per ml. ○, Type I interferon; ●, type I interferon plus gangliosides (52 μM sialic acid); □, type II interferon; ■, type II interferon plus gangliosides (52 μM sialic acid).

DISCUSSION

Data presented in this communication provide evidence that mouse type I and type II interferons differ in properties related to their biological activities. Both antiviral and antigrowth effects of type I interferon are inhibited by gangliosides, whereas those of type II are not; type I interferon binds to ganglioside affinity columns whereas type II interferon does not; L-1210R cells selected for resistance to mouse fibroblast interferon (14) respond to antiviral and antigrowth activities of type II interferon as well as do the L-1210 parent cells from which they are derived.

It is not known whether type I or type II interferon or parts of them have to enter target cells to cause inhibition of virus multiplication or cell growth. The facts that mouse type I interferon interacts with carbohydrate constituents of ganglioside molecules and that some transformed mouse cells gain increased sensitivity to its antiviral effect after uptake of exogenous gangliosides into the cell membrane (13) suggest that interaction of this type of interferon with cell membrane gangliosides is of functional significance. Clearly, if this is the case, then type II interferon must have a different mechanism by which it interacts with its target cells.

It is possible that there are two classes of interferon binding sites on the cell membrane of susceptible cells, each specific for productive interaction with only one type of interferon. Thus, prolonged culture of L-1210 cells in the presence of fibroblast interferon could select for those cells that have nonfunctional or no binding sites for fibroblast interferon but still carry unaltered sites for binding of type II interferon. Alternatively, uptake mechanisms for both interferons or their active fragments might be different, one involving gangliosides and the other involving a different type of glycolipid or none at all. Third, although the biological responses to both types of interferon appear to be identical, there might be different mechanisms by which these are triggered, involving activation of different enzymatic steps, each specific for one type of interferon. At the present time there is no direct evidence to

permit selection of the correct possibility. The recent observation that, in comparison to L-1210S cells, L-1210R cells are also less sensitive to plant toxins such as ricin (24), which is known to bind to gangliosides (25, 26), supports the hypothesis that lack of response of these cells to fibroblast interferon is a cell membrane-related phenomenon involving gangliosides.

There are two aspects of medical significance related to our observations. First, it is known that cancer patients often have increased levels of circulating gangliosides, which might reflect increased concentrations of these glycolipids in the tumor-surrounding tissue (27, 28). Therefore, treatment of such patients with type I interferon might not be effective because human leukocyte and fibroblast interferons also bind to gangliosides (12, 13). Our results suggest that treatment of such patients with human type II interferon would be an alternative in cases in which type I interferons fail to show the desired effects, provided that human type II interferon is comparable to the mouse variety regarding lack of affinity for gangliosides. Second, the observation by Gresser *et al.* (14) concerning the selection of fibroblast interferon-resistant leukemia cells might be of relevance to interferon therapy of leukemia patients, which likewise might select for resistant cells that would escape from the desired growth inhibition. Our data suggest that alternation between type I and type II interferons might be a useful approach to prevent such selection.

Note Added in Proof. The mouse immune interferon preparation used throughout these studies had the following characteristics which substantiate that the observed biological effects are, indeed, due to type II interferon activity. Over 90% of its antiviral activity was destroyed after treatment at pH 2 for 90 min at room temperature. It had no antiviral activity on human FS-4 cells at concentrations 20-fold higher than those that completely protected mouse L cells against EMV (assay 1). Furthermore, it had identical antiviral and antigrowth activities toward two different batches of L-1210R cells provided by Ion Gresser on two separate occasions. Both L-1210S and L-1210R cells revealed 95–99% viability after 3 days of culture in the presence of this interferon preparation. Using different preparations of mouse immune interferon prepared by the same procedure as the material used in this study, we have subsequently found that the degree of VSV inhibition in L-1210R cells by comparable interferon concentrations was variable in different batches and sometimes lower than in L-1210S cells. After this manuscript was submitted, an article (29) that described the antiviral effect of mouse immune interferon on another type I interferon-resistant mouse cell line appeared. This cell line (MSV-IF), obtained after prolonged culture in the presence of type I interferon, showed no antiviral response to type I interferon but was sensitive to type II interferon.

We thank Drs. Sonnenfeld and Merrigan for a sample of their type II interferon, Dr. Gresser for L-1210S and L-1210R cells, and Dr. Basu for G_{M2} and G_{M3} gangliosides. This work was supported by grants from the National Science Foundation (PCM 76-84125), the National Institutes of Health (AI-15007), and Institut National de la Santé et de la Recherche Médicale, France (78.4.082.1).

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