Interferon enhancement of the invasive capacity of Ewing sarcoma cells *in vitro*

(homogeneous interferon/basement membrane/type IV collagenase/metastasis/antiproliferative effect)

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ABSTRACT The ability of interferons to reduce cell proliferation *in vitro* and *in vivo* is a well-studied phenomenon. To extend such observations, the effect of interferons on the invasiveness *in vitro* of human malignant cells derived from a Ewing sarcoma was evaluated. Two related parameters were examined: (i) production of type IV (basement membrane) collagenase and (ii) penetration of human amnion basement membrane and collagenous stroma. After 6 days of treatment with crude fibroblast, leukocyte, or lymphoblastoid interferon at 100 units/ml in serum-free medium, type IV collagenase levels increased 2- to 4-fold per cell relative to those of untreated controls. With homogeneous fibroblast and lymphoblastoid interferons, a 2-fold elevation in type IV collagenase was detected after 2 days, with further increases, occasionally dramatic, occurring on the 4th and 6th day of treatment. The ability of Ewing sarcoma cells to invade human amnion connective tissue was measured after 6 days of treatment with various interferons. Relative to the behavior of untreated controls, crude leukocyte interferon, homogeneous lymphoblastoid interferon, and homogeneous fibroblast interferon at 100 units/ml augmented invasiveness 3-, 17- and 22-fold, respectively, when cells were allowed 4 days in which to traverse the amnion. When untreated cells were exposed simultaneously to the amnion and to homogeneous lymphoblastoid or fibroblast interferon, a 4- to 5-fold increase in invasiveness above control levels was observed in 2 days. These data emphasize the complexity of interferon-induced phenomena. In any overview, the effects of interferon on both the tumor cell and the host must be considered.

Although the antiproliferative activity of interferons both *in vivo* (1-3) and *in vitro* (4-7) is well documented in the human system, the direct effect of these substances on metastatic behavior has not been sufficiently explored. Previous studies of the effects of interferon on metastasis *in vivo* could not distinguish among effects on tumor cell intrinsic invasive behavior, on tumor cell proliferation, and on host immune responses (8, 9). Although an agent able to inhibit cell growth of a specific tumor type might, as a secondary effect, diminish metastasis of that tumor, in the general case, invasive behavior of human tumors does not necessarily correlate with their growth rate (10). We have approached these interrelated problems by examining the effects of several interferon preparations on the "invasive potential" of a defined population of human tumor cells. In this study, invasive potential has been evaluated *in vitro* in terms of two related parameters: (i) the amount of type IV (basement membrane) collagenase activity produced by cells during a fixed period and (ii) the rate at which cells can penetrate human amnion basement membrane and collagenous stroma. Previous studies have shown that the ability of a cell to degrade

type IV collagen *in vitro* correlates with the incidence of spontaneous lung metastases derived from a transplanted tumor of the same cell type *in vivo* (11). In general, a tumor cell must traverse basement membranes and interstitial connective tissue in order to metastasize. Our approach, therefore, measures phenomena crucial to one aspect of the complex metastatic process. The experiments reported here focus attention on the effects of interferon on the tumor rather than on the host. Our data indicate that both lymphoblastoid (α) and fibroblast (β) interferon-treated Ewing sarcoma cells exhibit markedly elevated levels of type IV collagenase and a dramatic enhancement in invasiveness *in vitro* relative to that of untreated controls. Crude and homogeneous interferons gave qualitatively equivalent results.

MATERIALS AND METHODS

Cell Culture Conditions. Ewing sarcoma cells, 5838B-Z, are routinely cultured at 37°C in 5% CO₂/95% air in 75-cm² Falcon tissue culture flasks in RPMI 1640 medium/10% heat-inactivated fetal bovine serum. However, serum-free culture conditions were used throughout the experiments described in this study. When the aim was to measure type IV collagenase levels in the culture fluids, the Ewing cells were trypsinized free of the Falcon flask in which they were growing, washed, and suspended at $\approx 10^5$ /ml in serum-free medium. Subsequently, 1 ml of cells was transferred to each well of a 24-compartment tissue culture dish and the cells were incubated overnight to allow them to attach. At zero time, the medium was removed and replaced with fresh medium in the presence or absence of interferon. Fresh solutions containing fresh interferon, where appropriate, were provided every other day. On days 2, 4, and 6, cell culture fluids were harvested and the number of viable cells was determined by using trypan blue exclusion as an indicator. When cells were grown for the purpose of measuring invasiveness, two techniques were used. In the first experimental design, cells were incubated in serum-free medium with and without interferon in 25-cm² Falcon tissue culture flasks. Culture fluids were replaced every other day. After 6 days, the cells were shaken free of the flask, counted, suspended at 5×10^5 /ml in serum-free medium in the absence of interferon, regardless of whether or not they had previously been treated with interferon, and transferred to the amnion. In the second experimental design, cells grown in serum-containing medium were trypsinized, washed, suspended at 5×10^5 /ml in serum-

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free medium in the presence or absence of interferon, and placed immediately on the amnion.

Collagenase Assays. Culture fluids from groups of 10^5 cells were centrifuged at 1,500 rpm for 5 min and stored at -20°C until all samples in a given experiment had been harvested. Type IV collagen-degrading activity was determined in an appropriate aliquot essentially as described by Liotta *et al.* (11). However, collagenase was not concentrated with ammonium sulfate before assay. To ensure that initial rates were obtained, the amount of soluble collagen produced was measured in most experiments after 6, 12, and 24 hr of digestion of ^{14}C -labeled type IV collagen (12). In all cases, data are expressed as radioactivity (cpm) solubilized per 10^5 cells per 24 hr. Duplicates were performed; results are expressed as averages. When cell death occurred, the number of viable cells at the beginning and end of each interval was averaged for subsequent normalization.

Preparation of the Amnion and Construction of the Chamber for Measurements of Invasiveness. Fresh human amnion basement membrane was denuded of epithelial cells as described (13) and placed in a Lucite chamber with the epithelial surface uppermost. The insertion of the amnion in the horizontal plane divided the chamber into two compartments. The design of the chamber and methods pertaining to its use have been detailed by Russo *et al.* (13). At zero time, 5×10^5 viable cells in serum-free medium in the presence or absence of interferon, were placed in the upper compartment of the chamber. Fresh serum-free medium with or without interferon was supplied every other day to both compartments. When interferon was included in the upper chamber, it was also added at the same concentration to the lower chamber. Cells capable of penetrating the denuded membrane together with the underlying stroma were trapped on a Millipore filter in the lower compartment after 2 or 4 days and quantified.

However, in the system used here, invading cells were captured on 8- μm Millipore filters and, after fixing both amnion and filter with 10% formalin, cells bound to the filter were visualized with hematoxylin/eosin stain. Separation of the amnion from the filter lying beneath it and the subsequent mounting of the filter remained unchanged.

Interferons and Interferon Assays. Crude leukocyte and fibroblast interferons were obtained from the Research Resources Branch, National Institute of Allergy and Infectious Diseases, Bethesda, MD. Crude lymphoblastoid interferon, a gift of K. C. Zoon, had a specific activity of $\approx 2 \times 10^5$ units/mg of protein. This material consisted solely of the α -component; the β -interferon component of lymphoblastoid interferon had been removed. Interferons were assayed by measuring inhibition of the cytopathic effect of encephalomyocarditis virus on GM2504 human fibroblasts trisomic for chromosome 21. Titers are expressed in international units based on the National Institutes of Health standard for leukocyte or fibroblast interferon (14).

Statistical Analysis of Invasiveness. The data were evaluated by using the Kruskal-Wallis analysis of variance by ranks (15). Values of *P* indicate the confidence limits within which each treatment differs from the relevant control.

RESULTS AND DISCUSSION

Characteristics of Ewing Sarcoma and Cultured Ewing Cells. Ewing sarcoma is a malignant childhood tumor that arises in bone and rarely in extraskelatal sites. This tumor is highly invasive and metastatic (16). The Ewing sarcoma cells (5838B-Z) (17) used for this study retained key characteristics of the parent tumor. When they were administered subcutaneously to the subscapular region of 3-wk-old athymic *nude* mice, tu-

mors developed, that, on microscopic examination after 6 wk, demonstrated features typical of Ewing sarcoma. Small uniform hyperchromatic tumor cells that were *P*-aminosalicylic acid positive and diastase sensitive (18) predominated.

Development of the Antiviral State. We first studied the ability of interferon to inhibit virus killing of Ewing sarcoma cells. Cultured Ewing cells were incubated for 18 hr with crude lymphoblastoid (α) or fibroblast (β) interferon at 100 units/ml and subsequently challenged with encephalomyocarditis virus. Details of the methodology are described elsewhere (14). The results indicated that the Ewing cells were able to respond to interferon by developing the antiviral state. The number of laboratory units of interferon/ml measured with Ewing cells was identical to the stated number of international units/ml as defined by the National Institutes of Health standard (data not shown). By way of comparison, these cells were roughly equivalent in sensitivity to HSF-4 fibroblasts challenged with vesicular stomatitis virus, a combination commonly used for determining interferon titers (19), and 1/20th as sensitive as human fibroblasts trisomic for chromosome 21. The latter cells are among the most sensitive known in the interferon system (20).

Effect of Crude and Homogeneous Interferons on Proliferation of Ewing Cells. Cells were cultured in the presence and absence of interferon for 6 days. On days 2, 4, and 6, the number of viable cells was determined. A typical set of data is given in Table 1. The interferons, regardless of type or concentration, had little or no systematic effect on cell growth during the first 4 days of treatment. Thereafter, the results depended strongly on the type and purity of the interferon used. Cells in the presence of crude leukocyte interferon (α) showed little proliferative activity throughout the 6-day period. In contrast, crude lymphoblastoid interferon, also an α -interferon, was toxic beyond 4 days of treatment at either 10 or 100 units/ml. Since homogeneous lymphoblastoid interferon was nontoxic and a slight increase in the number of viable cells occurred during the 6-day incubation with this material, the conclusion was drawn that the deleterious substances were contaminants and not the interferons themselves. Similarly, crude fibroblast interferon at 10 or 100 units/ml caused a decrease in viable cells between day 4 and day 6 of treatment whereas, in the presence of homogeneous fibroblast interferon, the number of cells remained virtually unchanged. Relative to untreated controls, two of which are shown in Table 1, the nontoxic interferons had a marginal antiproliferative effect. In the absence of interferon, cell numbers increased 1.6- to 1.9-fold in 6 days; in the presence of interferon, the largest increase was <1.4 -fold.

Table 1. Effect of interferons on cell growth

Interferon	Concentration, units/ml	Cells, no. $\times 10^{-5}$		
		Day 2	Day 4	Day 6
Homogeneous fibroblast	100	3.3	2.6	3.5
Homogeneous lymphoblastoid	100	2.5	2.5	3.4
Crude leukocyte	100	2.3	2.6	2.6
None		2.5	2.6	4.8
Crude lymphoblastoid	100	1.6	1.4	0.33
Crude fibroblast	100	1.8	2.4	0.56
Crude lymphoblastoid	10	1.9	1.4	0.72
Crude fibroblast	10	1.4	2.2	0.94
None		1.2	1.6	1.9

Ewing cells in serum-free medium were treated with various concentrations of interferon. Viable cells were determined by using trypan blue exclusion as an indicator. Fresh medium containing fresh interferon, where applicable, was provided every other day.

Effect of Crude and Homogeneous Interferons on Type IV Collagenase Levels. The amount of type IV collagenase secreted by Ewing cells in the presence and absence of various types of interferon was followed for 6 days. At the times indicated, the number of viable cells and the amount of collagenase elaborated by these cells during the preceding 2 days was determined. As shown in Fig. 1, measurements of the rate of appearance of type IV collagenase in the cell supernatants revealed no dramatic changes, relative to control levels, throughout 4 days of treatment with crude leukocyte interferon at 100 units/ml. Crude lymphoblastoid interferon and fibroblast interferon, each at the same concentration (100 units/ml), behaved similarly (data not shown). When similar measurements were made after 6 days of exposure to the crude interferons, the rate of collagenase production increased 2- to 4-fold relative to that of controls, depending on the nature and concentration of the crude interferon tested (Fig. 1B). Both crude lymphoblastoid and crude fibroblast interferon, each at 10 or 100 units/ml, stimulated type IV collagenase production. Treatment of Ewing cells with crude leukocyte interferon also resulted in increased levels of type IV collagenase. However, with the exception of crude leukocyte interferon, the impure materials were toxic during the interval between 4 and 6 days (see Table 1); hence, no absolute increases in collagenase levels were discerned in the culture fluids. In contrast, absolute increases in the rate of collagenase secretion in the presence of crude leukocyte inter-

FIG. 1. Type IV collagenase activity in the culture media from interferon-treated and untreated Ewing sarcoma cells. Cells were incubated in the presence and absence of various concentrations and types of interferon. Fresh medium and fresh interferon, where appropriate, were provided every other day. At the times indicated, culture fluids were harvested and viable cells were determined. At the termination of each experiment, type IV collagen-degrading activity in the culture fluids of all samples was determined. Cell numbers at the beginning and end of each 2-day period were averaged before normalizing the data. (A) Bars: a, untreated controls; b, crude leukocyte interferon at 100 units/ml; c, homogeneous fibroblast interferon at 100 units/ml; d, homogeneous lymphoblastoid interferon (M_r 18,500) at 100 units/ml. (B) Bars: a and b, as in A; e, crude fibroblast interferon at 100 units/ml; f, crude lymphoblastoid interferon at 100 units/ml; g, crude fibroblast interferon at 10 units/ml; h, crude lymphoblastoid interferon at 10 units/ml.

feron were easily demonstrated, relative to the levels of untreated controls, during the same period. Although these data suggested that interferon-treated cells produced higher amounts of type IV collagenase than their interferon-free counterparts, they also emphasized the need for further studies with purified interferons.

The results of similar experiments with homogeneous lymphoblastoid interferon (specific activity, 2 to 3×10^8 units/mg) and homogeneous fibroblast interferon (specific activity, 2 to 5×10^8 units/mg) are included in Fig. 1A. Small increases in type IV collagenase levels were discerned after 2 days of treatment with either of the homogeneous interferons and markedly elevated rates of appearance of type IV collagenase were evident after 4 days. Incubation of Ewing cells with each of the homogeneous interferons for 6 days resulted in striking increases in the rate of production of type IV collagenase. By using homogeneous interferons or crude leukocyte interferon, the stimulation of type IV collagenase could be dissociated from cell viability; unlike their impure counterparts, homogeneous lymphoblastoid and fibroblast interferons were capable of eliciting enhanced levels of the enzyme without demonstrable toxicity to the cells. These results establish that interferons, not their impurities, are the causative agents.

Effect of Crude and Homogeneous Interferons on Invasiveness. To ascertain whether the effect of interferons on type IV collagenase was indicative of an effect on the ability of cells to invade healthy tissue, a model system for the quantitative assessment of invasiveness was required. Such a system was constructed *in vitro* by using human amnion, divested of epithelial lining, as a barrier to the passage of cells (21). Amnion is made up of a basement membrane layer overlying a collagenous stroma (22). Cells able to traverse both basement membrane, the major structural component of which is type IV collagen, and the underlying stroma, composed of types I and III collagen as well as proteoglycans and hyaluronic acid (21), can be captured and quantified on the distal side of the amnion. This connective tissue matrix is impermeable to colloidal carbon and presents a mechanical obstruction to cell penetration. Enzymatic degradation of the matrix is thought to be necessary for tumor cell invasion. With the amnion assay, we compared the invasiveness of Ewing sarcoma cells that had been incubated for 6 days in the presence of various interferons at 100 units/ml as described in *Materials and Methods*. In each experiment, equal numbers of treated and control cells were subsequently suspended in fresh medium in the absence of interferon and applied to the amnion. Crude leukocyte interferon stimulated invasion of the amnion connective tissue matrix >3-fold relative to that of controls in a 2- or 4-day period (Table 2, experiment A). In contrast, cells treated with homogeneous lymphoblastoid or fibroblast interferon exhibited 17- and 22-fold increases in invasiveness, respectively, relative to control levels when cells were allowed 4 days in which to traverse the amnion. Statistical analysis performed by ranks showed that the difference between the control samples and the interferon-treated cells was highly significant. The failure of control cells to penetrate the amnion rapidly did not reflect a defect in their ability to attach (data not shown); both control and interferon-treated cells bound equally well to amnion basement membrane. Nevertheless, the ability of the treated cells to penetrate the matrix has been markedly altered. An increase in invasiveness with either crude lymphoblastoid or fibroblast interferon as the effector was also observed but, because degeneration of the cells during interferon treatment varied, subsequent measurements of invasion were considered to be unreliable. We therefore concentrated our efforts on the homogeneous interferons.

The enhanced invasiveness induced by interferon treatment

Table 2. Effect of interferons on invasion of human amnion basement membrane by Ewing sarcoma cells

Exp.	Interferon	Invasive colonies, % control	
		Day 2	Day 4
A	None	100 (10)	100 (8)
	Crude leukocyte*	340 (9)	310 (5)
	Homogeneous lymphoblastoid*	680 (5)	1,750 (6)
	Homogeneous fibroblast†	140 (2)	2,180 (4)
B	None	100 (6)	100 (6)
	Crude lymphoblastoid*	230 (3)	110 (2)
	Homogeneous lymphoblastoid	550 (3)	ND
	Homogeneous fibroblast*	420 (6)	480 (6)

Experiment A: Cells were treated with various interferons at 100 units/ml for 6 days in serum-free medium. Immediately thereafter, 5×10^5 viable cells, based on trypan blue exclusion, were suspended in fresh serum-free medium in the absence of interferon and transferred to the amnion. Invading cells adhering to the entire filter located in the lower compartment were counted. The number of invading colonies in cell samples not treated with interferon was arbitrarily fixed at 100%. The actual number of colonies was 77 ± 21 in 2 days and 194 ± 88 in 4 days. Interferon-treated invasive colonies are expressed as a percent of the average of controls using the same amnion. Each colony consisted of a single cell or a cell clump. Results are averages of separate experiments. Experiment B: Ewing cells, 5×10^5 per sample, were treated with various interferons at 100 units/ml in serum-free medium during incubation on the amnion. Interferon was present in both the upper and lower compartments of the chamber. Invasive colonies were determined as described for experiment A. Invading colonies in control samples were 41 ± 19 and 75 ± 20 , respectively, in 2 and 4 days. Values in parentheses represent numbers of determinations. ND, not done.

* $P < 0.005$.

† $P < 0.025$.

in vitro was confirmed in a second type of invasion assay (Table 2, experiment B). To decrease the time spent in serum-free medium, cells were allowed to invade basement membrane in the presence of homogeneous interferons without previous exposure to this material. Such an abbreviated experimental design also minimizes the effects of possible cell proliferation or cell death. Both of the homogeneous interferons stimulated invasiveness 4- to 5-fold after only 2 days of exposure to interferon. Where appraised, invasiveness after 4 days continued to increase relative to that of controls. This behavior was not evident in several experiments with crude lymphoblastoid interferon included for comparison. Treatment with the crude material resulted in a transient increase in invasiveness in 2 days that disappeared in 4 days, apparently owing to cell death. The results show that invasiveness in the abbreviated assay was very much less than that determined with the same concentration of homogeneous interferons using the design of experiment A. This observation is consistent with the measurements of type IV collagenase displayed in Fig. 1A: 2 days of interferon treatment are sufficient neither for the attainment of maximal collagenase levels nor for the establishment of maximal invasiveness.

Comparison of the data in Fig. 1 and Table 2 shows that elevated collagenase levels accompany increased invasiveness in a qualitative rather than a quantitative manner. Furthermore, an increase in invasiveness as a function of crude lymphoblastoid interferon treatment occurred before a change in collagenase level was detected (Table 2, experiment B). This relationship conforms to the intrinsic properties of each assay. In the first case, the amount of type IV collagen solubilized in a given period of time is a linear function of the amount of specific collagenase present. In the second case, invasiveness—the ability of cells to traverse the amnion—appears to be autocatalytic. A

small number of aggressive cells can produce a channel in the amnion through which less aggressive cells can pass. As the concentration of type IV collagenase in the medium increases, the possibility of passive invasion also increases. The autocatalytic nature of the assay may be further enhanced by the affinity of the amnion for type IV collagenase. Thus, a trivial increase in bulk collagenase, homogeneously distributed in solution in a collagenase assay, will become significant when concentrated at or near the connective tissue surface in an invasion assay. In support of this view, interferon-induced invasiveness 50- to 100-fold higher than control values has occasionally been observed with several interferons. (These data are not included in Table 2.) This marked rate of amnion penetration was not a result of either membrane breakage or aberrant collagenase levels. It most likely results from random juxtaposition of several aggressive cells that together create a passage or passages through the membrane. By quantifying invasive colonies, rather than cells, we have minimized the effect of passive transit through the amnion. Treating the data in this manner also serves to avoid overestimates stemming from the passage of single cells that divide on or after they have traversed the amnion. It should be noted, however, that the serum-free conditions used in these invasion experiments discourage but do not necessarily abolish cell division. Given the inherent differences between the nature of collagenase determinations and the nature of invasion assays, our data are consistent with a key role for type IV collagenase in the interferon-induced invasion process.

Interferon and Cancer—Some Interpretative Remarks. Interferon is a multipotent substance. It has been shown to possess both antiviral (23) and antiproliferative activities (1–7), attributes of profound clinical importance. Recently, an augmentation of plasminogen activator has been observed after exposure of macrophages to interferon (24). Here we show *in vitro* that interferon can also stimulate the invasive potential of cultured malignant cells under conditions completely divorced from its effect on cell proliferation. It should be emphasized that these results pertaining to the ability of interferon to stimulate type IV collagenase and to enhance invasiveness of Ewing sarcoma cells *in vitro* cannot *a priori* be extended to other cell types or tumors *in vivo*. Furthermore, the fact that interferon can stimulate host natural killer cell activity must be considered in any overview (25). Such stimulated natural killer cells could reduce an effect of interferon on tumor cell invasiveness *in vivo* by destroying blood-borne tumor emboli. However, in some experiments performed *in vitro*, treatment of selected target cells with interferon rendered them less sensitive to killing by natural killer cells (26). Only target cells sensitive to the antiviral effect of interferon were protected against cytotoxicity mediated by natural killer cells (27). Clearly, the relative importance of the effects of interferon on the tumor and on the host must be assessed for each tumor type. In view of the rapidity with which interferon is being introduced as a therapeutic tool, it is important to act quickly to broaden our understanding of this many faceted material in order to use it advantageously.

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