Effect of Fibroblast, Lymphoid, and Myeloid Interferons on Human Tumor Colony Formation in Vitro

Edward C. Bradley and Francis W. Ruscetti

ABSTRACT

A variety of solid and hematological human tumors and normal human bone marrow specimens were assayed for colony formation in a short-term soft-agar culture system. The effect of human fibroblast, lymphoid, and myeloid interferons on inhibition of colony formation was assessed. The effect of interferon on colony formation formed a continuum from complete inhibition to stimulation of growth. Of 40 evaluable tumor specimens, 18 showed at least a 70% inhibition of colony formation in the presence of interferon, at concentrations of 1000 units/ml or less. Four specimens (acute myelogenous leukemia, osteogenic sarcoma, neuroblastoma, ovarian carcinoma) showed at least 3-fold stimulation of colony formation with interferon. Two of 12 normal bone marrow specimens grown with colony-stimulating, factor-conditioned media showed greater than 70% colony inhibition with interferon. A dose-response relationship was seen in all tumor specimens tested. While fibroblast interferon was the most active in this system, all interferons showed the same magnitude and direction of activity. Continuous exposure and 1-hr incubation of tumor cells with interferon were identical in terms of colony inhibition. These data support the ability of this assay system to select tumors responsive in vitro to interferon, suggest the optimal species and concentration for inhibition or stimulation of growth, and support a direct role of interferon in the regulation of cell growth independent of other immunoregulatory actions of interferon. Such information may prove useful for predicting response in vivo to interferon in Phase II trials.

INTRODUCTION

Interferons are glycoproteins produced by almost all nucleated cells in vertebrates in response to a variety of stimuli (1). Interferons bind to receptors in the cell membrane and initiate a series of intracellular processes, including the de novo synthesis of novel intracellular enzymes; and alter a number of cellular functions, including the inhibition of translation of viral mRNA, net membrane charge, rate of cell division, and cell differentiation. Some of these interferon-mediated activities may be mechanisms responsible for antitumor properties which have been observed in vitro and in vivo (1, 8).

Many human tumors can be grown in short-term culture, and Salmon et al. (14) have shown a correlation between the inhibition of tumor colony formation in vitro by cytotoxic antitumor drugs and the in vivo response to those drugs. We undertook a survey of the effects of human leukocyte and fibroblast type (type I) and lymphoblastoid type (type II or immune) interferon on the formation of colonies in a soft-agar assay of 55 fresh and 10 cultured human tumors and 12 normal human bone marrows. Our results show that: (a) while colony formation is often inhibited by interferon, there is much variability of response to interferon within each tumor type; (b) a dose-response relationship and the relative activity of an interferon species can be determined; and (c) colony inhibition in this system may not require the presence of immunocompetent cells or their products and appears to be a direct, noncytotoxic effect of interferon.

MATERIALS AND METHODS

Source and Preparation of Human Tumor Cells

Fresh Human Tumor Specimens. The procedure for collecting, preparing, and culturing single-cell suspensions of human tumors has been described (9). All patients studied had histologically confirmed cancer and gave informed consent for a portion of a tumor mass, bone marrow, or ascites fluid which was being removed for diagnostic or therapeutic purposes to be used in this study. Most of the patients studied had received intensive chemotherapy prior to obtaining the tumor specimen. An aliquot of each specimen received was examined histologically to confirm its malignant nature. All ascites fluids were collected in sterile heparinized vacuum bottles and centrifuged at 100 x g for 10 min. The cells were then washed twice in RPMI-1640 with 10% FCS and forced through 25-gauge needles until a single-cell suspension was obtained. Bone marrow specimens were aspirated into syringes containing preservative-free heparin, suspended in RPMI-1640:FCS, layered over a Ficoll-Hypaque column, centrifuged at 1000 x g for 10 min to remove RBC, and washed twice in RPMI-1640:FCS. Normal bone marrow specimens were obtained by posterior iliac crest aspiration of young (mean age, 26 years) volunteers and were processed identically to the method for the malignant bone marrow specimens. Routine Wright-Giemsa and iron stains were performed on each donor aspirate and all were normal. Tumor biopsies were processed by prompt mechanical dissection of the tumor and serial passage through progressively smaller needles, until a single-cell suspension was obtained. Viability was measured by the ability of the cells in suspension to exclude trypan blue dye.

Human Tumor Cell Lines. Two osteogenic sarcoma cell lines (G-292, MG-63), 2 multiple myeloma lines (RPMI 8226, HS-Sultan), and a neuroblastoma line (IMR-32) were obtained from the American Type Culture Collection, Rockville, Md. A non-African Burkitt's lymphoma line, JL-P(C), was contributed by Dr. Ian McGrath, National Cancer Institute, Bethesda, Md. (5). Three cell lines derived from human myeloid leukemia speci-

1 To whom requests for reprints should be addressed, at Department of Medicine, Beth Israel Hospital, 330 Brookline Avenue, Boston, Mass. 02215. Received May 30, 1980; accepted October 9, 1980.

2 The abbreviations used are: RPMI-1640, Roswell Park Memorial Institute Medium 1640; FCS, fetal calf serum; CSF, colony-stimulating factor; CFU-c, colony-forming unit cell.
mens (HL-69, KG-1, and K-562), a human melanoma line (FCC-1), and a cutaneous T-cell lymphoma line (HUT-102) were also studied (4, 7, 11, 12). All cells were harvested for interferon assay in agar 48 to 72 hr after splitting the cultures and changing the media. Single-cell suspensions were prepared as described above and cells were washed twice in RPMI-1640: FCS prior to plating.

Source, Preparation, and Assay of Interferons

Purified human lymphoblastoid interferon was obtained from the Research Resources Branch, National Institute of Allergy and Infectious Diseases, Bethesda, Md. Human diploid fibroblast interferon purified approximately 10,000-fold by affinity chromatography to a specific activity of 1 × 10^6 interferon units/mg protein was obtained from HEM Research, Inc., Rockville, Md. Human myeloid interferon was obtained by infecting KG-1 human myeloid cells with Newcastle disease virus. The KG-1 cells were centrifuged at 1000 × g for 5 min and resuspended at 2 × 10^5 cells/ml in RPMI-1640:20% FCS with 4 × 10^4 hemagglutinin units of Newcastle disease virus per ml. After 24 hr incubation at 37°, the cells were removed by centrifugation at 1000 × g for 5 min. The supernatant was then ultracentrifuged at 36,000 × g for 2 hr. The supernatant was then collected and passed through a 0.22-μm filter (Millipore Corp., Bedford, Mass.). All interferons were assayed prior to use by HEM Research, Inc., utilizing the dye-uptake human foreskin-VSV method of Finter (6).

Growth of Tumor Cells in Agar

Cells were cultured using a 2-layer soft-agar system similar to that described by Hamburger and Salmon except that (a) mineral oil-primed BALB/c mouse spleen cell-conditioned media was omitted, (b) neither 2-mercaptoethanol nor DEAE-dextran was used in the cellular layer, and (c) transformin (2 μg/ml) was used in the cellular agar layer (9, 10). Briefly, underlayers of augmented McCoy’s Medium 5A (Grand Island Biological Co., Grand Island, N. Y.) and 0.5% agar (Difco Laboratories, Inc., Detroit, Mich.) were prepared in 35-mm Petri dishes. Cells to be assayed were suspended in 0.3% agar in augmented Connaught Medical Research Laboratories Medium 1066 (Grand Island Biological Co.) with 15% horse serum (Flow Laboratories, Rockville, Md.) and plated over the McCoy’s agar underlayer. All specimens were plated in triplicate.

Use of CSF in Normal Bone Marrow Assays. Normal donor bone marrow cells were cultured in 2 different media. One was identical to that used for malignant marrow. The other was similar, except that supernatant from a human trophoblastic cell line transformed with sarcoma virus was added (3). This conditioned medium has colony-stimulating activity, producing 60 to 80 colonies/liter × 10^5 normal bone marrow cells in a standard CFU-c assay. Interferon was then added to both CSF-containing and standard, nonconditioned plating media as described below.

Use of Interferons in the Growth Assay. In all specimens assayed, the interferon species to be tested was prepared as a solution of 10,000 IU/ml in RPMI-1640. The interferon was added to the final plating mixture just prior to plating at a concentration of 100 units/ml of plating mixture. In some specimens, other interferon concentrations were used to develop a dose-response curve. Single-cell suspensions of 2
tumor cell specimens were incubated for 1 hr at 37° in either RPMI-1640:FCS or RPMI-1640:FCS plus fibroblast interferon, 100 IU/ml. Cells were then washed twice in RPMI-1640:FCS, counted, assessed for viability, and plated as described above.

Assessing Growth of Colonies. All plates were incubated at 37° in an atmosphere of 6% carbon dioxide:air with 100% humidity. Plates were examined immediately after plating to assure that a single-cell suspension in agar had been achieved. Plates were examined twice weekly for colony growth and counted at 7 and 14 days using an inverted microscope. Cluster formation (aggregates of 5 to 49 cells) was seen on Days 3 to 5 and was maximal on Days 7 and 8. Colony formation of both treated plates and controls (a colony is defined as a new round aggregate of 50 or more cells) was seen from Days 5 to 10 and was maximal by Day 14. Disintegration of colonies was not seen until Day 21. All data presented represent the number of colonies counted on Day 14. The techniques for histological examination of colonies grown in agar have been described (9, 13, 14). Most colonies were examined by aspirating single colonies from the cellular agar layer with a Pasteur pipet, suspending the cells in RPMI-1640, and preparing a cytocentrifuged slide. Cells were then stained with Wright-Giemsa stain and examined microscopically. Some cellular agar layers were fixed in glutaraldehyde, transferred to a glass microscope slide, and stained with Wright-Giemsa or hematoxylin-eosin (13).

Assessing Evaluability and Statistical Analysis of Data. Each colony inhibition assay was plated in triplicate, and the standard deviation and standard error of the mean were determined for each group of 3 plates. All standard errors were less than or equal to 16% of the mean. Tumor specimens were considered evaluable if the mean number of colonies per control plate was at least 30. This definition of evaluability has been used in other studies referenced and was chosen prior to the analysis of our data.

RESULTS

Inhibition of Colony Formation by Interferon

Solid Tumors. Thirty solid tumors, 26 fresh specimens and 4 human lines, were assayed. Of these, 5 fresh specimens (1 breast adenocarcinoma, 1 chondrosarcoma, and 3 ovarian carcinomas) failed to form at least 30 colonies per control plate and were therefore not evaluable. The colony inhibition of evaluable specimens is presented in Table 1. Eight of 25 specimens (4 of 10 ovarians, 2 of 5 melanomas, 1 of 2 osteogenic sarcomas, and 1 of 1 chondrosarcoma) showed a 70% or greater colony inhibition. Seven specimens (1 of 5 melanomas, 1 of 1 rhabdomyosarcoma, 2 of 10 ovarian carcinomas, 1 of 1 testicular carcinoma, 1 of 2 breast carcinomas, and 1 of 1 lung adenocarcinoma) had an inhibition of colony formation from 35 to 69%, and 6 specimens (3 of 10 ovarian, 1 of 2 breast, 2 of 5 melanoma) showed less than 30% inhibition. Three specimens (1 of 10 ovarian, 1 of 2 osteosarcomas, 1 of 1 neuroblastoma) showed a 3- to 5-fold increase in the number of colonies with interferon. Of the 3 specimens in which interferon stimulated colony formation, 1 ovarian was a fresh tumor specimen and 2 (neuroblastoma, osteosarcoma) were cultured tumor lines. Fibroblast interferon showed the greatest activity in inhibiting colony formation, but the magnitude and the direction of interferon-induced change in colony formation was similar for all 3 interferons tested.
Hematological Tumors. Twenty-nine fresh hematological tumor specimens (9 bone marrow and 20 blood specimens obtained by leukapheresis) were assayed. Five of 9 bone marrow aspirates (2 of 3 chronic myelogenous leukemias, 3 of 6 acute myelogenous leukemias, 1 of 1 myeloma) and 4 peripheral blood specimens (3 of 14 acute myelogenous leukemias, 1 of 3 chronic myelogenous leukemias) were evaluable. Five of the 9 evaluable specimens (1 of 3 chronic myelogenous leukemias, 3 of 5 acute myelogenous leukemias, 1 of 1 myeloma) had at least a 70% inhibition of colony formation. Six hematological tumor lines were assayed and all were evaluable. Four lines (2 of 2 myelomas, 2 of 3 leukemias) had a 70% or greater inhibition of colony formation. One line (cutaneous T-cell lymphoma) showed no inhibition, and one leukemia line (K-562) showed a 3-fold stimulation of colony formation (Table 2). All interferon species showed similar magnitudes of colony inhibition or stimulation.

Normal Bone Marrow and Peripheral Blood Specimens. Twelve normal donor marrow aspirates obtained from healthy young volunteers were assayed. None of them formed colonies in the absence of CSF. All of them formed more than 30 colonies per control plate with CSF. With both fibroblast interferon and CSF in the plating media, 2 of 12 showed greater than 70% inhibition, 9 showed approximately 50% inhibition, and 1 showed no inhibition of colony formation. All colonies, irrespective of size, were counted equally, but the colonies in the interferon-treated plates tended to be smaller. No comparison of the relative degree of granulocytic maturity in interferon-treated and untreated plates was made. Four peripheral blood specimens from normal donors were assayed, but none produced enough colonies even in the presence of CSF to be evaluable.

Dose-Response Relationship between Colony Formation and Interferon

Concentration. Dose-response relationships were looked for in 6 fresh solid tumors and 1 multiple myeloma line. One melanoma specimen showed no inhibition of colony formation at concentrations up to 8000 units/ml, and a second showed only 10% inhibition at the same interferon concentration. The rest showed progressive inhibition with increasing interferon concentrations, and the myeloma line Sultan was completely inhibited at a concentration of 1000 units/ml. Representative dose-inhibition curves are presented (Chart 1).

Comparison of 1-Hr Exposure or Continuous Exposure to Interferon

Two human tumor specimens, a myeloma line and a fresh ovarian carcinoma, were exposed to interferon for 1 hr, washed of the interferon, and then plated. A control aliquot of each specimen was plated without interferon, and another aliquot was plated with interferon (continuous exposure) as described above. Incubation for 1 hr with interferon did not decrease the viability of the cells as measured by the ability to exclude trypan blue dye. The cells which had been exposed to interferon for only 1 hr showed the same degree of inhibition as those cells exposed continuously to the same concentration of interferon. In addition, a portion of the cells incubated with interferon for 1 hr and then washed were plated with interferon in the plating...
Effect of Interferon on Colony Formation in Vitro

Table 2

<table>
<thead>
<tr>
<th>Histology</th>
<th>Source</th>
<th>Control</th>
<th>Fibroblast interferon</th>
<th>Lymphoblast interferon</th>
<th>Leukocyte interferon</th>
<th>% of inhibition or stimulation of fibroblast interferon</th>
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<td>102</td>
<td>77% inhibition</td>
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<td>Human tumor cell lines</td>
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Table 3

Comparison of 1-hr incubation and continuous exposure of tumor cells to interferon and the inhibition of colony formation

<table>
<thead>
<tr>
<th>Assay conditions</th>
<th>Cells assayed for myeloma (ATCC-8226)</th>
<th>Ovarian carcinoma from fresh ascites</th>
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<tr>
<td>Incubated for 1 hr without HFI, washed, plated without HFI in media</td>
<td>5200 ± 368</td>
<td>629 ± 32</td>
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<td>Incubated without HFI, washed, plated with HFI in media</td>
<td>535 ± 39</td>
<td>232 ± 17</td>
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<tr>
<td>Incubated with HFI for 1 hr, washed, plated without HFI in media</td>
<td>490 ± 41</td>
<td>254 ± 31</td>
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<tr>
<td>Incubated with HFI for 1 hr, washed, plated with HFI in media</td>
<td>528 ± 37</td>
<td>219 ± 11</td>
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</table>

*HFI, human fibroblast interferon, 1000 IU/ml.

DISCUSSION

Fifty-five fresh human tumors, 10 tumor cell lines, and 12 normal bone marrow specimens were assayed for the effect of interferon on colony formation. The fresh solid tumors and malignant marrow specimens, tumor lines, and normal marrow in the presence of exogenous CSF readily formed evaluable numbers of colonies in vitro. The peripheral blood specimens obtained by leukapheresis from patients with leukemia formed colonies in only 4 of 20 specimens assayed. While all of the assayed peripheral blood specimens had morphologically immature circulating cells, it is possible that cells in the bone marrow are functionally more capable of forming colonies in agar than were morphologically similar cells circulating in the peripheral blood. It is also possible that other populations of cells in the bone marrow produce factors which are necessary for colony formation by the circulating malignant cells. Leukapheresis alone may alter the colony-forming ability of cells in the peripheral blood, and normal donor WBC obtained by leukapheresis formed no colonies in agar in the absence of...
CSF and fewer than 5 per control plate in the presence of CSF. This is consistent with the number of CFU-c’s observed in methylcellulose assay systems studying peripheral blood. Our data support the observation that growth in this agar culture system is characteristic of, but not limited to, malignant tissue (2, 16).

The response of colony formation to the addition of interferon formed a continuum from complete inhibition to five-fold stimulation. In our study, 7 of 21 fresh solid tumors and 5 of 9 fresh hematological malignant specimens had at least a 70% inhibition of colony formation with interferon. Some of the inhibited tumors, such as melanoma or gastric carcinoma, are notoriously resistant, both clinically and in this in vitro system, to standard cytototoxic antitumor agents. The concurrent in vivo use of interferon with this in vitro assay system could show whether or not colony inhibition is predictive for clinical response.

The effect of interferon on colony formation formed a continuum from complete inhibition of growth to stimulation of growth. In addition, the magnitude and direction of the effect on colony formation appear to be related to the individual tumor, not to the histological tumor types. For example, of 10 ovarian carcinoma specimens obtained mostly from malignant ascites, 4 showed at least a 70% inhibition, 2 showed essentially no inhibition, and 1 specimen, surprisingly, showed a 3-fold stimulation of colony formation. Similarly, of 4 fresh melanoma specimens, 2 showed marked inhibition and 2 showed no inhibition at all, even at interferon concentrations of 8000 IU/ml. Of the 2 osteogenic sarcoma lines tested, one showed an 83% inhibition and the other showed a 3-fold augmentation of colony formation. It is clear than one cannot predict, on the basis of cell type alone, what the response to interferon will be.

In addition to one fresh ovarian specimen and one osteosarcoma line, 2 other tumor cell lines showed an increase in colony formation in the presence of interferon. Both K-562 leukemia cells and IMR-32 neuroblastoma cells reproducibly formed more colonies in the presence of interferon. What the response to interferon will be.

Fourteen specimens were assayed with more than one interferon species. Each specimen showed the same magnitude and direction (inhibition or augmentation) of effect on colony formation. A positive dose-response relationship was seen in all tested specimens.

Very little is known about the mechanism of regulation of cell growth and differentiation by interferons. It is unlikely that nonspecific cytotoxicity is the sole reason for inhibition of colony formation. No decrease in cell viability was seen after short-term exposure to interferon, even though marked inhibition of colony formation was seen in the same cells, and no difference in inhibition was seen between cells exposed for 1 hr or exposed continuously to interferon. Some specimens showed marked inhibition at low interferon levels, some showed no inhibition at all levels of 8000 IU/ml. An occasional specimen had increased colony formation with interferon. This makes a general, cytotoxic effect unlikely as the explanation for the activity of interferon in this system. It is also unlikely that the inhibition is mediated by the activation of immunological mechanisms. Although interferon has been shown to influence immune function by enhancing natural killer cell activity, stimulating macrophage activity, and possibly enhancing the antigenicity of target cell (1, 15), the absence of immune competent cells or their products in a number of assays using cell lines sensitive to interferon makes immune enhancement as a mechanism of colony inhibition unlikely. Verma et al. (17) have shown that CFU-c number decreases and colony size decreases with exposure to interferon. With increasing interferon concentrations and decreasing colony size, they have shown an increasing percentage of immature cells in the colonies. While speculation about the mechanism of growth stimulation by interferon is beyond the scope of this discussion, if such a phenomenon occurs in vivo it could provide a growth advantage to the stimulated clone of malignant cells.

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