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Activity of Human Leukocyte Interferon in a Human Tumor Cloning System

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Summary. *Clinical trials using interferon to treat human malignancies are currently hampered by limited supplies of the compound. We have utilized a human tumor cloning system as an assay for the antitumor effects of human leukocyte interferon. Interferon was tested against 62 patients' tumors growing in this soft agar system. A dose-dependent cytotoxic effect of interferon was noted against only five of the patients' tumors. A ≥ 70% decrease in tumor colony-forming units (TCFUs) was noted with one lymphosarcoma cell leukemia, one small cell lung cancer, one adenocarcinoma of the lung, one breast cancer, and a pancreatic cancer. One patient had his tumor cultured in vitro and had a clinical trial with interferon. This patient whose tumor demonstrated in vitro sensitivity had a clinical antitumor effect with interferon therapy. The in vitro results in this study suggest that the human leukocyte interferon currently available has a low level of activity in a human tumor cloning system. Additional testing is needed to determine whether the cloning system can identify the patient(s) who might have an antitumor effect from the interferon.*

Introduction

Interferon has demonstrated inhibitory effects on a wide variety of experimental animal tumors [7]. In addition, early clinical trials with human leukocyte interferon have revealed some antitumor effects in selected patients with non-Hodgkin's lymphoma [9, 17], acute lymphocytic leukemia [13, 14], multiple myeloma [9, 16, 18], breast cancer [2, 9], malignant melanoma [15], and Hodgkin's disease [1].

These preliminary clinical studies have stirred considerable interest in interferon, and the compound deserves additional clinical trials. However, at present there are three major problems that have limited investigation with interferon. First, it has been difficult and expensive to manufacture large quantities of interferon; secondly, the currently available interferon preparations are impure; and finally, the type of interferon with the most potent antitumor activity has not yet been defined.

In the present study, we have utilized the recently developed human tumor cloning system of Hamburger and Salmon [10, 11] to determine the antitumor effect of interferon. This human cloning system has been shown to be predictive for response or lack of response of various malignancies to chemotherapy [21, 22, 26]. The human tumor cloning system might be useful in identifying patients who would benefit from interferon therapy. In addition, the system might provide information about the concentration required to achieve optimal cell kill. These pieces of information could allow the best utilization of the limited interferon resources. Finally, the assay might be helpful in comparing the effects of the various interferon preparations against human tumors in vitro without the necessity for huge randomized clinical trials.

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Patients and Methods

After obtaining informed consent according to federal regulations, we performed a total of 29 bone marrow aspirations, 53 tumor biopsies, 14 paracenteses, and 10 thoracenteses in 106 patients with advanced malignancies as part of routine diagnostic and follow-up studies. One-half of each specimen was sent for routine pathology studies, and the other half was sent for human tumor cloning studies.

Bone marrow cells were obtained by iliac crest puncture. Cells were aspirated into a syringe containing preservative-free heparin (100 U/ml). After centrifugation at 150 *g* for 10 min, the cells in the buffy coat were harvested with a pasteur pipette and washed twice

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in Hank's balanced salt solution (Grand Island Biological Co., Grand Island, NY) with 10% heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, NY). Cells from pleural fluid and ascites were prepared in a similar manner. Solid tumors obtained immediately after surgery were mechanically dissociated under aseptic conditions. These tissues were minced with a scalpel, teased apart with needles, passed through 20, 22, and 25 gauge needles, and then washed by centrifugation. The viability of bone marrow, fluid, and solid tumor specimens was determined in a hemocytometer with trypan blue. Viability was routinely more than 90% for marrow and fluid specimens and 16%–50% for the solid tumor specimens.

Cells were cultured as described by Hamburger and Salmon [10, 11]. Cells to be tested were suspended in 0.3% agar in enriched CMRL 1066 medium (Grand Island Biological Co.) supplemented with 15% horse serum, penicillin (100 U/ml), streptomycin (2 mg/ml), glutamine (2 m*M*), $CaCl_2$(4 m*M*), and insulin (3 U/ml). Just prior to plating, asparagine (0.6 mg/ml), DEAE-dextran (0.5 mg/ml) (Pharmacia Fine Chemical, Division of Pharmacia, Inc., Piscutaway, NJ), freshly prepared 2-mercaptoethanol (final concentration 50 m*M*), and human leukocyte interferon (Research Resources Branch, National Institute of Allergy and Infectious Diseases) were added to the cells at final concentrations of 50, 500, and 1,000 U/ml. One milliliter of the resultant mixture was pipetted onto 1 ml feeder layers in 35 mm plastic petri dishes. The final concentration of cells in each culture was 5×10^5 cells in 1 ml agar-medium-interferon mixture. The bottom feeder layer of this two-layer soft agar system consisted of McCoy's 5A medium plus 15% heat-inactivated fetal calf serum and a variety of nutrients as described by Pike and Robinson [19]. Immediately before use, 10 ml 3% tryptic soy broth (Grand Island Biological Co.), 0.6 ml asparagine, and 0.3 ml DEAE dextran were added to 40 ml enriched medium. Agar (0.5% final concentration) was added to the enriched medium and underlayers were poured in 35 mm petri dishes. All plating was done in triplicate.

After preparation of both bottom and top layers, cultures were incubated at 37° C in a 7.5% CO_2 humidified atmosphere. Colony counts (≥ 50 cells constituted a colony) were made on days 10 and 20 after plating. To determine the decrease in tumor colony-forming units (TCFUs) caused by the cytotoxic interferon, counts of colonies growing from interferon-treated cells were compared with counts of colonies growing from cells incubated with saline instead of interferon. A decrease in TCFUs by ≥ 70% in the interferon plates was utilized as a definition of a response in vitro [26].

Patient 1. One patient whose tumor was cultured in the human tumor cloning system was also treated with human leukocyte interferon. The patient was a 50-year-old man with null cell lymphosarcoma cell leukemia. He had been previously treated with cyclophosphamide, Adriamycin, vincristine, and prednisone (CHOP) for one cycle. When the disease progressed, he began treatment with Adriamycin, vincristine, cytosine arabinoside, and prednisone (AdOAP), again without response. He then started therapy with methane sulfonamide-(4-(9-acridinylamino)-3-methoxyphenyl)-(m-AMSA) with no response. A bone marrow specimen was sent for human tumor cloning at a time when he had massive lymphadenopathy, and a peripheral white blood count was 85,000 with 86% lymphoma cells. Assay of the patient's tumor revealed 0% decrease in TCFUs with Adriamycin, 0% decrease with chlorambucil, 5% decrease with vincristine, 0% decrease with m-AMSA, and an 85% decrease in TCFUs with 1,000 U human leukocyte interferon/ml. Because of the 85% decrease in TCFUs with the interferon in culture the patient was prescribed daily doses of human leukocyte interferon at doses of 3×10^6 U/day. Serum levels of interferon were not obtained in this patient but this dose of interferon has been shown to give serum interferon titers of 100–140 U/ml serum in patients with breast cancer [9]. Because of the absence of any antitumor effect, and indications from the clonogenic assay that larger doses were needed, the dose of interferon was escalated to 9×10^6 U/day. On that regimen the patient's lymphadenopathy regressed to less than half its former size and remained decreased for 6 weeks. Peripheral blood counts decreased, but circulating blasts remained high despite leukophoresis. The patient was finally referred for total body radiotherapy because of a progressive increase in size of the lymph nodes.

Results

In vitro Sensitivity Studies

Sixty-two of the 106 patients' tumors cultured formed colonies in culture. Evidence that these colonies were indeed tumor and not granulocyte-macrophage colonies or fibroblasts has been presented previously [9, 10, 11, 12, 24–26]. Table 1 identifies the 62 malignancies grown in culture. All these malignancies formed at least 30 colonies per plate. The number of tumors in which the interferon caused a ≥ 70% decrease in TCFUs (at a concentration of 1,000 U/ml) was only five (8%). These tumors, which were called sensitive in vitro, included one lymphosarcoma cell leukemia, one small cell lung cancer, one adenocarcinoma of the lung, one breast cancer, and one pancreatic cancer. There were three patients' tumors in which interferon caused an increase in number of colonies over control growth. These included one adenocarcinoma of lung (82% increase in number of

Table 1. Number of in vitro responses to human leukocyte interferon in the human tumor cloning assay

Tumor type	No. cultured	No. with ≥ 70% decrease in TCFUs[a]
Breast	23	1
Melanoma	6	0
Ovary	5	0
Lung		
Adenocarcinoma	4	1
Small cell	5	1
Lymphoma (non-Hodgkin's)	6	0
Neuroblastoma	3	0
Lymphosarcoma cell leukemia	2	1
Pancreas	2	1
Multiple myeloma	2	0
Renal	1	0
Colon	1	0
Stomach	1	0
Osteogenic sarcoma	1	0

[a] At a concentration on 1,000 units of human leukocyte interferon/ml

colonies) and two ovarian cancers (39% and 141% increase, respectively).

Twenty-one of the 62 patients had interferon dose-response studies performed on their tumors. Figure 1 details the percent of colonies surviving for these twenty-one patients' tumors tested at each of three concentrations of interferon. As shown in Fig. 1, interferon had no or minimal effect on 15 different patient's tumors in vitro even at concentrations as high as 1,000 U/ml (patients 2, 3, 4, 6, 7, 8, 9, 11, 12, 13, 16, 18, 19, 20, 21). Only patient 1 (lymphosarcoma cell leukemia), patient 14 (small cell lung cancer), and patient 15 (pancreatic carcinoma) had $\geq 70\%$ decrease in TCFUs at the highest concentration of interferon utilized (1,000 U/ml).

Clinical Studies

One patient whose tumor was tested in vitro was also treated clinically with interferon. Patient 1, described above, had lymphosarcoma cell leukemia., At 1,000 U interferon/ml, there was an 85% decrease in the number of colonies formed in vitro. Clinically, the patient had an antitumor effect from the interferon at very high doses of the compound. This antitumor effect was confined to a decrease in the size of the lymph nodes. The patient could not, however, be said to have experienced a partial remission, because of continued circulating leukemic blast cells.

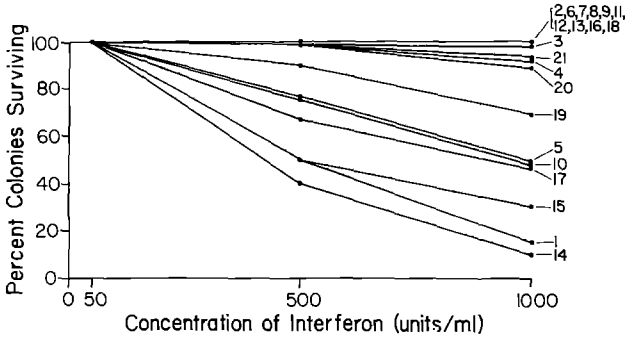

Fig. 1. Effect of human leukocyte interferon on survival of colonies growing in human tumor cloning assay. Numbers at end of each *line* correspond to patient numbers. Each *point* represents a mean percent of colonies surviving for three plates with standard errors of ± 15%. Colony counts were performed on day 20. Patient 1, lymphosarcoma cell leukemia; patient 2, osteogenic sarcoma; patient 3, ovarian cancer; patients 4, 5, 6, neuroblastoma; patients 7, 8, 9, breast cancer; patients 10, 11, 12, 13, 14, small cell lung cancer; patient 15, pancreatic cancer; patient 16, melanoma; patient 17, multiple myeloma; patients 18, 19, adenocarcinoma of the lung; patient 20, diffuse histiocytic lymphoma; patient 21, adenocarcinoma of unknown origin

Discussion

This study has utilized a human tumor cloning system as an assay for the in vitro antitumor activity of human leukocyte interferon. The first finding is that human leukocyte interferon appears to have an effect ($\geq 70\%$ decrease in TCFUs) on the TCFUs of a small percentage (5 of 62 or 8%) of the human tumors tested. Other investigators, including Epstein et al. [5, 6], Bradley and Ruscetti [4], and Salmon [20], have previously reported an in vitro effect of human leukocyte interferon in the human tumor cloning system. They noted in vitro activity against ovarian cancer [4–6, 20] and other malignancies, including adenocarcinoma of the lung [4, 20], breast cancer [4, 20], prostate cancer [20], and others [4]. This finding in a relatively simple in vitro system supports the concept that interferon could conceivably inhibit tumor growth by a direct cytotoxic effect on tumor cells and not by an indirect mechanism. One must remember, however, that there are cells of the immune system on the plate, such as lymphocytes or macrophages, which could mediate the decrease in TCFUs.

Interferon preparations have been shown to inhibit a number of human tumor cell lines growing in monolayer and suspension cultures [2, 7], as well as to inhibit colony formation of these cell lines growing in agarose [8]. It is important to remember, however, that interferon has not been completely purified. It is possible that some antitumor effects observed with crude interferon preparations are due to a factor (or factors) other than interferon [7].

The second finding of the present study is that the colony-forming units of the human tumors are heterogeneous in their sensitivity to interferon in much the same manner as they are heterogeneous in their sensitivity to standard anticancer agents [21, 22, 26]. Only 1 of the 23 breast cancers tested in vitro responded ($\geq 70\%$ decrease in TCFUs) to the interferon preparation. There were three specimens in which there were actual increases in the number of colonies on interfron plates versus control plates. This heterogeneity was also noted by Bradley and Ruscetti [4].

It is of interest that there was a dose-response effect of interferon against some tumors in the present study. This may have some therapeutic implications. Larger doses of interferon may be needed to effect a clinical response in certain patients receiving the compound. The in vitro dose of 1,000 U/ml utilized in this study is very high and is difficult to achieve with the presently available preparations of human leukocyte interferon. A more purified preparation of interferon may improve this situation.

Of all the tumor types tested in vitro, the only in vitro activity (≥ 70% decrease in TCFUs) of interferon was noted against lymphosarcoma cell leukemia, small cell lung cancer, breast cancer, adenocarcinoma of the lung, and pancreatic carcinoma. Clinical responses to interferon have previously been reported in patients with leukemia and breast cancer. It may now be important to explore the clinical activity of the compound in small cell lung cancer, pancreatic cancer, and adenocarcinoma of the lung. However, it should be reemphasized that the in vitro activity for interferon in these tumor types in the present study was actually quite low and unremarkable when compared with the activity of standard antineoplastics.

Unfortunately, only one patient to date has had his tumor assayed with interferon in the human tumor cloning system and also had a clinical trial with the compound. This patient, with refractory lymphosarcoma cell leukemia, had a response in the clonogenic assay and had definite shrinkage of tumor-involved lymph nodes. Thus, in this clinical trial, the human tumor cloning assay correlated with the clinical effects of interferon therapy. Obviously it is not possible to conclude that the human tumor cloning assay will be an accurate guide for the use of interferon. However, the results suggest that further clinical trials with interferon should compare the clinical results with the in vitro results of the human tumor cloning system. The ability to preselect patients likely to respond to interferon for clinical trials with the agent would be highly desirable and would provide maximal utilization of limited interferon supplies.

In the future the human tumor cloning assay may prove useful in screening the various types of interferon to find the type with the most significant antitumor effect. Performing these comparisons in the culture plate could preclude large randomized trials and conserve patient resources and interferon supplies.

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