Cytotoxic Effects of Interferon in Vitro on Granulocytic Progenitor Cells

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SUMMARY

We have utilized in vitro marrow culture techniques to evaluate the cytotoxicity for granulocytic progenitor cells of two highly purified human leukocyte interferon preparations. Concentration- and time-related decrements in granulocytic colony-forming capacity in agar occurred with human and mouse marrow. Although mouse marrow cells were less sensitive than were human cells, these data indicate lack of strict species specificity for the cell growth-inhibitory effects of interferon. Similar cytotoxicity was noted for normal and leukemic human clonogenic cells exposed to interferon for prolonged periods. The decrease in the proportion of granulocytic progenitor cells in DNA synthesis, which occurred at high concentrations, and the diminution by interferon of the cytotoxicity caused by cytosine arabinoside demonstrate that interferon decreases DNA synthesis of granulocytic progenitor cells. The lack of enhanced cytotoxicity for rapidly proliferating mouse post-endotoxin marrow cells indicates that interferon is not a cell cycle-stage-specific drug. These data seem useful for evaluating the suppressive effects of interferon on granulopoiesis and for devising clinical trials with this agent.

INTRODUCTION

Interferon is a virus-inhibitory protein produced by intact animals and cultured cells infected with viruses or stimulated with certain nonviral substances (6, 22). In addition to inhibiting viral replication, preparations of interferon also interfere with the proliferation of a variety of normal and neoplastic cells (21, 24). Based on encouraging results in experimental animal models, clinical trials utilizing interferon for treating a variety of normal and neoplastic cells have been organized (17, 31, 32, 36). During 1 of these trials (17), neutropenia was observed, which suggested possible suppression of granulopoiesis by interferon.

In vitro marrow culture techniques have been utilized to evaluate granulopoiesis in animals and human subjects (19, 35). With these methods marrow GPC develop colonies of myeloid cells in semisolid agar under the necessary influence of the stimulatory substance(s) termed CSA. Prior studies with these techniques have shown that in vitro cytotoxicity with certain chemotherapeutic agents correlated well with their in vivo effects (5, 12, 18, 20). Nonhuman interferon preparations have been shown to be inhibitory in vitro for mouse marrow granulocytic colony-forming cell proliferation (29). To improve our understanding of the effects of interferon, we have used in vitro marrow culture techniques to determine the cytotoxicity for human and mouse granulocytic precursors of P-IF preparations. Since interferon may be used in combination with other chemotherapeutic agents, we have evaluated the interaction of interferon with the cycle-active drug, ara-C.

MATERIALS AND METHODS

In Vitro Marrow Culture. Our methods for evaluating human and mouse marrow GPC in agar gel culture and the thymidine suicide technique, which determines the proportion of GPC-S, have previously been described (18). Human or mouse marrow cells were incubated in single-layer culture for determining CFC, with colonies of aggregates of >50 cells. For our assay of human CFC, we used human leukocyte-conditioned medium (at 15% concentration) as a source of CSA. Leukocyte-conditioned medium was prepared by incubating 1 to 2 x 10⁸ Hypaque-Ficol-separated mononuclear cells per ml in modified McCoy's Medium 5A containing 15% fetal calf serum and 0.5 mM 2-mercaptoethanol. For assay of mouse marrow CFC, mouse L-cell-conditioned medium (at 15% concentration) was used as a source of CSA. The L-cell-conditioned medium was prepared by modification of a previously described method (1). Fifteen ml of fresh complete medium were added to near-confluent layers of L-cells (approximately 2 x 10⁶ cells) in 75-sq cm tissue culture flasks (Falcon Plastics, Oxnard, Calif.). After 7 days of incubation at 37° in an air-CO₂ humidified atmosphere, the supernatants from both types of cell cultures were separately harvested, filtered, and stored at -20° prior to use as conditioned media. Batches of conditioned media maintained their CSA for at least 8 months of storage.

Experiments were designed to test the effects of interferon and ara-C, separately and in combination, on the

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3 The abbreviations used are: GPC, granulocytic progenitor cells; CSA, colony-stimulating activity; P-IF, partially purified human leukocyte interferon; ara-C, 1-β-D-arabinofuranosylcytosine (cytosine arabinoside, Cyto- sorin); CFC, colony-forming capacity; GPC-S, granulocytic progenitor cells in DNA synthesis; IA-IF, immunoabsorbed human leukocyte interferon.
survival of colony-forming cells. For short-term exposure 2 to 5 x 10^6 marrow cells per ml medium were placed in an air-CO_2 incubator at 37°C without drug (control cells) or with different drug concentrations for 1 or 4 hr. After exposure to the drug, the cells were washed 3 times with medium and were plated in agar; CFC was determined after 7 to 10 days of incubation. Surviving CFC's in cultures exposed to drugs were compared to those cultured simultaneously for the same time without drugs.

Suspension culture conditions were adequate for survival of colony-forming cells since 98 ± 6.1% (S.E.) of these cells unexposed to drug survived for the period of suspension culture. Cellular recovery was also comparable in the incubated exposed and unexposed cell suspensions. For evaluation of the cytotoxic effects of prolonged exposure of interferon and/or ara-C, these drugs were added directly to culture dishes prior to addition of the marrow cells in agar medium. Pilot experiments showed that the bulk of the antiviral activity of interferon (tested as described below) persisted in liquid medium at 37°C for the 10-day period of marrow culture. Prior studies have demonstrated the same persistence of ara-C in culture (20). Statistical analyses were performed with Student's t test.

Interferon Preparations and Assays. Two human leukocyte interferon preparations were evaluated. Partially purified interferon (P-IF), produced by Sendai virus stimulation of buffy-coat leukocytes according to the method of Cantell (7), had a specific activity of 5 x 10^8 units/mg protein. A more highly purified material (IA-IF) obtained by interferon antibody affinity chromatography processing of P-IF (2) had a specific activity of 2 to 3 x 10^9 units/mg protein and was provided by Dr. Kurt Paucker, Medical College of Pennsylvania, Philadelphia, Pa. The activity of the interferon preparations was assayed by measuring the ability of the preparations to inhibit plaque formation of vesicular stomatitis virus in human foreskin fibroblast cultures, as previously described (30). Interferon unitage is expressed in terms of Reference Standard 69/19 supplied by the National Institute of Allergy and Infectious Diseases, Bethesda, Md. One unit of human interferon in this assay is equal to 1 unit of the human interferon reference standard.

Interferon Inactivation. Interferon (P-IF) was inactivated by 2 methods and tested for antiviral effects as well as for its ability to inhibit granulocytic colony formation. The interferon was incubated with trypsin (0.1 mg/ml; Difco Laboratories, Inc., Detroit, Mich.) for 1 hr at 37°C before addition of ovalbumin or soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, Mo.). Trypsin inhibitor alone did not alter the antiviral or granulocyte-inhibitory effects of interferon. Rabbit anti-human leukocyte interferon globulin (Antiserum 24), prepared as previously described (2), was provided by Dr. George Galasso, NIH. The interferon preparation was incubated with the antiserum for 1 hr at 37°C in unit ratios of 1:0.5 to 1 for the neutralization experiments.

ara-C, ara-C (The Upjohn Co., Kalamazoo, Mich.) was dissolved in 0.9% NaCl solution and used after fresh preparation.

Endotoxin. In certain experiments mice were pretreated with endotoxin [20 μg i.p. (Lipopolysaccharide B, Salmonella typhimurium; Difco)] 24 hr before the marrow cells were collected and exposed to interferon in vitro. Endotoxin exposure increases the proportion of GPC-S and the in vivo and in vitro sensitivity of GPC to cycle-specific chemotherapeutic drugs (12, 20).

The Limulus lysate test, which detects the presence of endotoxin or endotoxin-like substances in biological materials (10, 27), was utilized to assess the presence of endotoxin in the interferon preparations. We utilized Pyrotel (Difco), a standardized reagent prepared from lysed amoebocytes of Limulus polyphemus, to detect endotoxin. Positive controls (Pyrotel, Difco) contained known concentrations of bacterial endotoxin. Test samples or positive and negative controls were incubated, as previously described (10, 27), with equal volumes of Pyrotel for 1 hr at 37°C. The formation of a gel in the test vial indicated the presence of endotoxin.

Patients. Interferon and ara-C effects were evaluated on marrow cells from 10 control subjects with morphologically normal marrow granulopoiesis and normal complete blood counts and from 3 patients with chronic myeloid leukemia. Marrow aspirations were performed after informed consent was obtained.

RESULTS

Human Marrow Cells. Continuous exposure of human marrow cells to both interferon preparations (P-IF and IA-IF) for 7 days produced essentially identical dose-related decrements in colony-forming cell survival (Chart 1). Marrow cells from patients with chronic myeloid leukemia showed similar susceptibility to the cytotoxic effects of 7-day exposure to interferon. Short exposures (1 or 4 hr) to P-IF interferon caused no decrease in normal colony-forming cell survival.

The effects of 1-hr exposure of human marrow cells to the combination of ara-C (1 μg/ml) and P-IF interferon (10^9 units/ml) in differing sequences were evaluated. Additive cytotoxicity (Chart 2) was noted when interferon was given during or after ara-C exposure but not when interferon preceded ara-C.

Prolonged exposure of human marrow cells to interferon and ara-C produced additive cytotoxicity at low levels of ara-C (Chart 3), particularly at the ara-C concentration of 0.0001 μg/ml (p < 0.05).

Mouse Marrow Cells. Continuous exposure of mouse marrow cells to both interferon preparations showed dose-related decrements in colony-forming cell survival (Chart 4). The inhibitory effects of P-IF and IA-IF were similar except for increased cytotoxicity by IA-IF at the relatively high concentration of 10^9 units/ml (p < 0.01). Mouse marrow colony-forming cells were less sensitive than were human cells to cytotoxic effects of both human interferon preparations, with a significant difference being found with P-IF (p < 0.001 by the Spearman-Karber analysis) (Charts 1 and 4). Marrow cells from postendotoxin mice (i.e., with a higher proportion of GPC-S (12, 20)) showed no increased sensitivity to interferon, and at concentrations ≥10^9 units/ml the cells showed decreased cytotoxicity (p < 0.05). Short exposure (1 hr) of mouse marrow cells to interferon was associated with minimal decrease in colony-forming cell survival (Chart 4). For further evaluation of the effects of interferon...
on cell cycle characteristics, thymidine suicide experiments were performed before and after 1-hr exposure of mouse cells to interferon. The proportion of GPC-S remained essentially unaltered from basal levels of 35.8 ± 2.8% with interferon concentrations of 10^2 and 10^3 units/ml. However, at concentrations of 10^4 units/ml the proportion of GPC-S decreased significantly to 22.9 ± 5.6% (p < 0.05).

Inactivation of the antiviral and nonantiviral activities of P-IF was performed by 2 methods to help determine what specificity of the cell growth-inhibitory effects was due to interferon. Trypsin treatment and anti-interferon globulin incubation inactivated the suppressive effects of interferon on human and mouse colony-forming cell proliferation (Table 1). Antibody neutralization (with rabbit anti-human interferon globulin) of interferon for determining mouse marrow colony formation could not be evaluated since normal rabbit serum markedly suppressed mouse CFC. These procedures also inactivated the antiviral effects of interferon as indicated by the diminution of antiviral titers with trypsinization and interferon antibody incubation from 3 x 10^3 to <30 units/ml and from 1.2 x 10^3 to <30 units/ml, respectively. The antiviral titer is the reciprocal of the level at which 50% of the viral plaques were inhibited (30).

Endotoxin in concentrations ≥0.1 µg/ml in vitro suppresses marrow CFC, whereas concentrations ≤0.01 µg/ml do not (9). The P-IF and IA-IF interferon preparations were assayed for the presence of endotoxin with the Limulus lysate test. At interferon concentrations of 10^2 to 10^3 units/ml (concentrations causing appreciable CFC cytotoxicity), the IA-IF contained no endotoxin, whereas the P-IF preparation was negative at 10^2 units/ml and contained <0.01 µg/ml endotoxin at 10^3 units/ml.

Since similar cells (human peripheral leukocytes) are used to produce the interferon preparations tested and the conditioned medium that we utilized for human marrow cell CSA, we assayed the interferon preparations for CSA. P-IF and IA-IF concentrations of 10^3 to 10^4 units/ml contained no CSA when added to human marrow cells cultured in agar, as described in "Materials and Methods."

DISCUSSION

Our data have demonstrated that prolonged exposure to 2 highly purified human leukocyte interferon preparations caused dose-related inhibition of the proliferation of both human and mouse marrow GPC. Although lesser effects were noted in the heterologous species, these findings indicate the lack of strict species specificity for the antiproliferative effects of human leukocyte interferon for marrow col-
Granulopoietic Cytotoxicity of Interferon araC alone

Chart 3. Cytotoxicity in vitro of prolonged exposure (7 day) of ara-C alone and in combination with interferon (Int), 50 units/ml, for human marrow colony-forming cells. Bars, S.E.

Chart 4. Cytotoxicity in vitro of interferon for mouse marrow colony-forming cells. Exposure times noted are: P-IF interferon, 1 hr and 10 days, postendotoxin (β endotoxin) marrow cells, 10 days; IA-IF interferon, 10 days, Bars, S.E.

Our findings of persistence of granulopoietic suppression with the more highly purified IA-IF interferon and of elimination of interferon cytotoxicity by trypsin and antibody inactivation of antiviral activity suggest that the myeloid cell growth- and viral-inhibitory effects were due to the interferon rather than the contaminating protein. Recent studies (3, 24, 26, 34) that have evaluated the electrophoretic profiles of interferon have provided strong evidence that both antiviral and cell growth-inhibitory activities are intrinsic properties of interferon. Our studies with the Limulus lysate test indicated that possible contamination of the interferon preparations with low concentrations of endotoxin did not contribute to the in vitro cytotoxicity of interferon for GPC. In addition, no CSA was present in the interferon preparations.

Endotoxin administration, which causes regeneration of marrow cells and increases the proportion of GPC-S (12, 20), did not enhance the suppressive effects of interferon on granulocytic proliferation, which suggests that interferon does not act as a cycle-specific agent. Short exposures of interferon at high dose levels decreased the proportion of GPC-S. Furthermore, interferon diminished the ability by short exposure of the cycle-specific agent, ara-C, to kill colony-forming cells (Chart 2). These findings indicate that short exposure of interferon, although not directly cytotoxic, interferes with granulocytic precursor cell cycling characteristics by decreasing the proportion of GPC-S.
Prior studies (11, 28) have shown that interferon inhibits cellular DNA synthesis. Prolonged incubation of marrow cells with both interferon and ara-C showed additive cytotoxic effects of interferon (Chart 3), which suggests transience of the interferon-induced suppression of DNA synthesis or effects of interferon during other stages of the cell cycle.

The cytotoxic effects of prolonged exposure (7 days) to interferon for granulocytic precursors from patients with chronic myeloid leukemia were similar to those for normal human cells. Cytogenetic studies have shown that colonies from patients with chronic myeloid leukemia derive from the leukemic line (33). Prior studies from our laboratory with these techniques have shown that cytotoxic effects also occur with concentrations of ara-C in vitro comparable to those that cause in vivo myelosuppression (15, 18, 20, 23). Thus, the in vitro suppressive effects of interferon for clonogenic leukemic cells suggest that this agent may prove to be useful in chemotherapeutic regimens for leukemia.

The concentration of interferon that we utilized in vitro and that showed cytotoxic effects correlate well with blood levels obtained after clinical administration of interferon (8, 13, 25). Our data show inhibition of in vitro granulopoiesis by interferon provides experimental evidence that may explain the neutropenia that develops following prolonged administration of interferon (17). The interferon concentrations achieved and the time course for development and resolution of the neutropenia are consistent with this thesis (Ref. 17; T. C. Merigan, unpublished observations). Similar cytotoxicity for granulocytic precursors by endogenous interferon elaboration may contribute to the neutropenia associated with viral infections. In this regard a granulocytic colony-inhibiting factor with properties similar to those of interferon has been generated in mouse serum after inoculation with the known interferon inducer, poly(I-C) (14). Furthermore, recent studies (4) have suggested the in vivo role of interferon in inhibiting marrow CFC and granulopoiesis in viremic experimental animals that develop neutropenia. The relationship between interferon and the lipoprotein inhibitors of granulopoiesis appearing in the serum of patients during viral infections (16) remains to be determined.

These studies appear to be useful for understanding the inhibitory effects of interferon on the proliferation of granulopoietic cells and should provide improved models for devising chemotherapeutic regimens with this agent for clinical trials.

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