Abrogation of Interferon-induced Resistance to Interferon-activated Major Histocompatibility Complex-unrestricted Killers by Treatment of a Melanoma Cell Line with 5-Fluorouracil

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ABSTRACT

Advanced cancer responds clinically to combined therapy with recombinant interferon-α and 5-fluorouracil. Although the two agents may interact in the biosynthetic pathway for thymidine, we investigated, as an alternative mechanism, the regulation of susceptibility of the A375 human melanoma to natural killers activated by interferon. A375 were preincubated with 5-fluorouracil, interferon, or both sequentially prior to assay as targets for cell-mediated killing. Pretreatment of A375 with interferon decreased apparent lytic efficiency. 5-Fluorouracil alone increased the susceptibility of A375 to killing. Pretreatment of targets with 5-fluorouracil abrogated the resistance normally induced by interferon pretreatment. Thus, 5-fluorouracil modulates certain immunoregulatory effects of interferon-α. Thymidine does not block the effect of 5-fluorouracil. While fluorodeoxyuridine is relatively ineffective in this system, fluorouridine is more effective than 5-fluorouracil in abrogating the effect of interferon. These data suggest important interactions of 5-fluorouracil and interferon in pathways for protein synthesis. It is known that interferon both increases the activity of natural killers and increases resistance of tumors to natural killers. We have shown that 5-fluorouracil, by blocking the resistance, may allow the augmented natural killing to be effective. This observation provides an alternate hypothesis for the clinical activity of 5-fluorouracil and interferon in combination.

INTRODUCTION

The usefulness of combining cytotoxic chemotherapy with biologics such as IFN in the treatment of solid tumors has recently been demonstrated by Wadler et al. (1). They observed a 76% response rate among patients with advanced colon cancer initially treated with the combination of IFN and 5-FUra and 43% among all patients. In subsequent investigations others have confirmed substantial, although lesser, activity of this combination in colon cancer (2, 3) and in other malignancies (4). The combined response rate among 113 patients treated in the three studies of colon cancer is 34%. We recently reported a 44% response rate among a small number of patients treated with a similar regimen (5).

Synergistic antiproliferative interaction of IFN with 5-FUra and its analogues has been shown in cell culture systems. Namba et al. (6) showed that continuous exposure of several human cancer cell lines produced additive or synergistic inhibitory effects in five of seven instances. Welander et al. (7) failed to show an additive or synergistic interaction of IFN and 5-FUra in a clonogenic assay using the human ovarian adenocarcinoma cell line BG-1. Elias and Crissman (8) evaluated a murine adenocarcinoma and a human myeloid leukemia. They found a synergistic interaction in inhibiting proliferation. The synergistic interaction of IFN and 5-FUra was substantially more striking with the myeloid cell line than with the adenocarcinoma cell line. Wadler et al. (9, 10) tested the human colon cancer cell lines HT-29 and SW480. They showed significant enhancement of the cytotoxicity of 5-FUra by IFN.

IFN has significant immunomodulatory effects in addition to its antiviral and antiproliferative effects. There is a substantial literature demonstrating that IFN increases the cytolytic activity of natural killer cells (11-15). This effect may be observed with purified lytic populations treated with IFN in vitro as well as with natural killers harvested from patients being treated with IFN. IFN also increases the resistance of tumor targets to lysis by natural killers (16, 17). It follows from these various lines of evidence that IFN may have countervailing effects in vivo, with an activation of cytolytic mechanisms coupled with the induction of tumor cell resistance to these same mechanisms.

It has been demonstrated that the target cell resistance induced by IFN may be abrogated by pretreatment of the target cells with cycloheximide, an inhibitor of protein synthesis (16). This raises the possibility that other antimetabolites, such as 5-FUra and its analogues, with inhibitory activities in the protein synthetic pathways, might also affect the induction of resistance by IFN. In the studies presented here, we explored the possibility that the clinical activity of the combination of 5-FUra and IFN in colon cancer, reported by Wadler et al., may not be related to modulation by IFN of the antitumor effects of 5-FUra. We assume that IFN activates natural killers in these patients and propose that 5-FUra blocks the induction by IFN of resistance of the patients’ tumors to natural killers. Therefore, we tested the hypothesis that 5-FUra, used in vitro in clinically relevant concentrations, is able to block the IFN-induced resistance of tumor targets to killing by IFN-activated natural killers.

MATERIALS AND METHODS

Cell Lines. The malignant melanoma cell line, A375, was obtained from the American Type Culture Collection. It was carried as a monolayer in tissue culture flasks in Dulbecco’s modified Eagle’s medium (Whitaker Bioproducts, Walkersville, MD) supplemented with glutamine, penicillin/streptomycin mixture, and 10% fetal calf serum. Lines were tested periodically for Mycoplasma (Gen-Probe; Gen-Probe Inc., San Diego, CA) and found to be uninfected.

Killer Cells. Peripheral blood was collected by venipuncture from healthy, young adult laboratory workers. Mononuclear cells were collected by flotation on Ficoll-Hypaque and washed. The populations were both depleted of adherent cells and activated by overnight incubation in flat bottom, tissue culture plastic with culture medium (RPMI 1640 with antibiotics, glutamine, and 10% fetal calf serum) containing recombinant IFN-α at a concentration of 100-1000 IU/ml. IFN-activated killers resulting from this procedure possess the following characteristics: (a) by flow cytometry, all gated cells are 6 ± 1% (SEM) CD14+; lymphocytes 92 ± 1% CD2+, 79 ± 2% CD3+, 23 ± 4% CD8+, 14 ± 1% CD16+, 14 ± 2% CD56+, 9 ± 1% CD16+ CD56+, 2 ± 1% CD3+ CD56+; (b) vigorous depletion of adherent cells markedly reduces the proportion of CD14+ cells yet increases killing; (c)
lysis is not restricted by HLA antigens since a wide variety of human targets is efficiently killed; (d) CD25 (receptor for interleukin 2) is expressed at low levels (5 ± 1%); (e) preincubation of targets with w6/32 ascites, an antibody specific for HLA class I framework enhances killing. These properties, combined with the property of enhanced activity after stimulation with IFN-α or IFN-γ, strongly resemble those of IFN-stimulated natural killers (18).

Assay of Killers. A375 cells in a subconfluent condition were incubated from 1 to 2 h with Na2CrO4. The monolayer was washed, harvested with trypsin-EDTA, washed three more times, and counted. Targets were plated in a 96-well round bottom microtiter plate in a volume of 100 µl containing 5000 targets. Killer cells were added in multiplicities of 1- to 100-fold in a volume of 100 µl. Each condition was assayed in triplicate. Maximum release of radioisotope was determined by addition to targets of 100 µl of 0.1 N HCl. Spontaneous release was determined by addition of 100 µl of assay medium. Plates were centrifuged at 75 x g for 2 min and incubated for 3–16 h. The Titercult Supernant Collection System was used to harvest the cultures, and the samples were counted in a gamma counter. Percentage of specific lysis was calculated as

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\text{Experimental release - spontaneous release} \div \text{Maximum release} \div \text{Experimental release} \times 100%.
\]

Lytic units were calculated by performing a linear regression on the titration of the corrected percentage of lysis as a function of the number of killer cells on a log scale. A lytic unit is defined as the number of killers required to achieve 30% lysis of 5000 target cells. "Lytic units," as displayed in the figures, refers to concentration of lytic activity and is calculated as the number of lytic units/million harvested and activated peripheral blood mononuclear cells.

Pretreatment of Targets. A375 monolayers were washed and incubated in fresh medium containing 5-fluorouracil at concentrations of 10^{-8}-10^{-6} µg/ml for 1–20 h. Where appropriate, they were then washed and incubated with IFN (rIFN-α2b, Intron A; Schering, Kenilworth, NJ) at concentrations of 1–10,000 IU/ml for 16 h. Cells to be used in a cytotoxic assay were then washed and labeled as described above.

Statistics. A linear regression of percentage of specific lysis on log (number of killers) was calculated for all conditions in an experiment. Lines were compared by testing the X intercept (19), on the simplifying assumption that the lines were parallel. Computations used Procedure GLM of the SAS system.

RESULTS

Activation of Killers. Mononuclear cells, derived from peripheral blood and depleted of plastic-adherent cells by overnight incubation, kill A375 melanoma at low levels. The same cells, when incubated overnight in IFN, acquire greatly increased lytic efficiency. As shown in Fig. 1, IFN at 10 units/ml more than doubles killing capacity, and 1000 units/ml increases killing manyfold.

Resistance to Killers Induced by Interferon. It has been known that IFN pretreatment of targets induces resistance to killing mediated by natural killers and other related types of killers. It is demonstrated in Fig. 2 that A375 melanoma becomes resistant to adherent cell-depleted natural killers with a reduction in apparent lytic efficiency of at least 50%. IFN-activated killers are more efficient in killing A375, but IFN pretreatment of A375 also results in resistance to killing. Susceptibility and resistance are relative, since more efficient killers partially neutralize or override the resistance demonstrated against less efficient killers.

Induction of resistance follows a shallow dose-response curve, with resistance seen at 10 units/ml for 16 h and maximal at 100–1000 units/ml. Significant resistance occurred in 8 of 11 consecutive experiments in which the targets were treated with the clinically achievable concentration, 100 units/ml, and the killers with 100 or 1000 units/ml.
Effect of 5-FUra on Killing of A375. A375 melanoma was incubated in 5-FUra at graded concentrations prior to assay as targets with IFN-activated killers. Preincubation of targets with 5-FUra for 1 h (Fig. 3A) has only a very modest effect. It is evident in Fig. 3B that spontaneous cell death, seen in the absence of added killer cells, begins to increase at about 1 Ìg/ml exposure for 20 h. Increased specific lysis, dependent on added killers and measured over spontaneous release, is also seen at 1–10 Ìg/ml. Calculated lytic units increase approximately 3-fold when targets are preincubated with 5-FUra at 10 Ìg/ml.

Abrogation of Resistance by 5-FUra. 5-FUra treatment of targets prior to IFN treatment prevents the IFN-induced resistance. As shown in Fig. 4, 5-FUra increases lysis mediated by IFN-activated killers (Δ). At concentrations >1 Ìg/ml, 5-FUra blocks the reduction in lysis mediated by IFN (□). The 5-FUra effect is both time and dose dependent. Fig. 5 shows targets pretreated with 5-FUra at graded concentrations for various times. Each tower represents the lytic units observed for IFN-activated killers against targets treated with IFN after 5-FUra as a percentage of the lytic units observed with targets treated with medium after 5-FUra. The front row is a single comparison of targets treated with IFN but not with 5-FUra; IFN induces a >50% reduction in lysis on the basis of lytic units. As opposed to abrogation of resistance, targets often become “supersensi-

Fig. 4. Abrogation of IFN-induced resistance by pretreatment of targets with 5-FUra for 20 h. Targets were pretreated with graded doses of 5-FUra from 10⁻³ to 10 Ìg/ml. □, preincubation with 5-FUra only; Δ, 5-FUra followed by IFN at 100 units/ml for 16 h. IFN effect: at 5-FUra = 0, 0.01, 0.1, and 0.3, P < 0.0001; at 5-FUra = 1.0, P < 0.002; at 5-FUra = 3.0 and 10.0, nonsignificant. 5-FU, 5-FUra.

Mechanism of 5-FUra Effect. A major pathway for cytotoxic effects of 5-FUra involves the formation of a slowly reversible ternary complex of thymidylate synthase, 5-FUra, and a methyl donor. Inhibition of synthesis of thymidine results, and inhibition of synthesis of DNA results, in turn. Addition of preformed thymidine to the culture medium blocks any 5-FUra effect based on inhibition of thymidylate synthase (20).

Comparison of the interaction of 5-FUra and IFN in thymidine-free and in thymidine-rich medium demonstrates that thymidine is without effect (Fig. 6). IFN in the absence of 5-FUra promotes resistance of targets and thymidine does not alter this effect. Pretreatment of targets with 5-FUra results both in increased susceptibility to lysis and in abrogation of IFN-induced resistance; thymidine does not alter either phenomenon. These data indicate the 5-FUra effects are not mediated through inhibition of thymidylate synthase.
cell carcinoma and melanoma (21-23). IFN mediates a variety of tumor targets. An interaction between an inhibitor of protein synthesis and IFN has now been extended to 5-FUra.

Confirmation of this conclusion resulted from experiments with 5-FUra analogues (Fig. 7). FdUrd, a thymidine precursor which directs the 5-FUra effect to the DNA synthetic pathway, is without effect upon IFN-induced resistance. FUrd, a false base in the pathway for synthesis of RNA, exhibits a contrasting effect with highly efficient abrogation of resistance induced by IFN.

**DISCUSSION**

5-FUra and IFN interact in modulating cell-mediated lysis of tumor targets. An interaction between an inhibitor of protein synthesis and IFN has previously been shown and this observation has now been extended to 5-FUra.

IFN has shown disappointing activity as an anticancer agent for solid tumors, although activity has been reported in renal cell carcinoma and melanoma (21-23). IFN mediates a variety of immunological effects with potential antitumor activity, and such effects have been demonstrated in vivo (12, 14-15, 24). No convincing correlation between immunomodulatory effects in patients and responses of solid tumor patients has ever been demonstrated. The phenomenon of IFN-induced resistance to lytic mechanisms may explain the failure to make this correlation. Since 5-FUra abrogates the resistance, the combination of 5-FUra and IFN may lead to tumor responses by an immunological mechanism. This hypothesis merits further exploration.

The concentrations of IFN used in these experiments ranged from 100 to 1000 units/ml. These concentrations are comparable to peak levels of IFN achievable in vivo after s.c. or i.m. administration (25). 5-FUra has been administered in a variety of doses and schedules. The bolus administration frequently used in combination with IFN results in a peak concentration of 10-100 µg/ml with a half-life of only a few minutes (20). Continuous infusion of 5-FUra results in a steady state level of 0.1-0.5 µg/ml. The peak level achieved with administration of 5-FUra by i.v. bolus is comparable to the concentrations of 5-FUra required in these studies. However, the steady state level of 5-FUra achieved clinically would not be effective in our system if limited to a 20-h exposure time. Longer exposure was not tested in this system.

The Wadler regimen should be considered in terms of an "induction phase" of infusional 5-FUra plus IFN and a "maintenance phase" of weekly 5-FUra plus IFN. The contrasting pharmacokinetics for 5-FUra in the two phases support the assumption that the biochemical interaction of the two agents may also differ markedly, regardless of the bioassay used. In the study by Wadler et al. (1), median time to response was 6 weeks; in the study by Kemeny et al. (2), 7 of 9 responses occurred by 12 weeks; in the study by Pazdur et al. (3), 14 of 15 responses occurred at 8 weeks, the earliest observation point. Ajani et al. (26) reported a poorly effective regimen with 5-FUra by bolus only and IFN-γ. These considerations suggest that the induction phase defined above might be the active component. In any case, each phase must be considered separately in attempts to define mechanisms or to optimize regimen.

The leading hypothesis concerning IFN-induced resistance of targets to cell-mediated lysis concerns the expression of class I histocompatibility antigens. IFN induces increased expression of these antigens on the cell surface. Several lines of evidence relate increased expression of these antigens to decreased susceptibility to killing by natural killers. This evidence includes comparative susceptibility to lysis of (a) variant targets differing in the intensity of expression of class I antigens (27) and (b) target pairs with differing levels of antigen expression resulting from gene transfection in one member of the pair (28). Increased expression of histocompatibility antigens may require increased protein synthesis. Experiments with cycloheximide suggest that resistance to lysis requires protein synthesis (16). These considerations suggest the postulate that 5-FUra may block resistance by blocking protein synthesis rather than by inhibiting DNA synthesis.

5-FUra interferes with cellular metabolism in three primary ways. (a) It inhibits DNA synthesis by binding tightly to the enzyme thymidylate synthase. This mechanism is enhanced by leucovorin. The same mechanism is the primary mode of action of the 5-FUra analogue FdUrd. (b) It may be incorporated into DNA and interfere with DNA replication. (c) It is converted to a false ribonucleotide easily incorporated into RNA and interferes in this way with protein synthesis.

Chu et al. (29) have proposed that IFN-γ blocks reactive synthesis of thymidylate synthase. This effect would explain synergy with 5-FUra through the pathway of DNA synthesis. Their observation leads to the predictions that (a) leucovorin would enhance the 5-FUra + IFN synergy, (b) thymidine would
block the synergy in vitro, (c) FdUrd would be at least as effective in producing synergy as 5-FUra. Elias and Crissman have demonstrated the second and third predictions to be true in vitro. Their data contrast with ours, since they observed inhibition of the interaction by thymidine and greater interactive efficacy of FdUrd than of FdUrd with IFN. No published clinical data bear on these predictions yet.

If a single mechanism mediates the clinically important antitumor effect of 5-FUra + IFN, then clinical studies comparing 5-FUra + IFN with 5-FUra + leucovorin + IFN will tend to distinguish between the hypothesis of direct cytotoxicity and the hypothesis of complex immunological modulation. We predict, to the extent that the immunological interaction contributes to regression of tumor, that leucovorin will not enhance the interaction of 5-FUra with IFN clinically. Furthermore, we predict that FdUrd will not interact effectively with IFN, since FdUrd has little effect on protein synthesis.

If 5-FUra interacts with IFN to modulate sensitivity of tumor targets to killers, may it not also counteract IFN in the stimulation of natural killers? 5-FUra suppresses various aspects of immune competence in animal models including the availability of precursors of natural killers in mice (30). 5-FUra failed to predict, to the extent that the immunological interaction contributes, to regression of tumor, that leucovorin will not enhance the interaction of 5-FUra with IFN clinically. Furthermore, we predict that FdUrd will not interact effectively with IFN, since FdUrd has little effect on protein synthesis.

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