Continued Malignant Cell Proliferation in Head and Neck Tumors during Cytotoxic Therapy

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ABSTRACT

The effect of cytotoxic therapy on the proliferation of squamous cell carcinoma of the head and neck in vivo in patients was evaluated before and 15–35 days after the start of therapy. To accomplish this, iododeoxyuridine was administered at t = 0, and bromodeoxyuridine was administered 15–35 days later during treatment with a tumor biopsy obtained for study immediately after each pyrimidine infusion. Monoclonal antibodies specific for the halogenated pyrimidines were used to identify cells that were in the S-phase at the time of the infusions. Eleven patients were studied prior to treatment. Of those, the intratreatment biopsy of eight patients contained tumor tissue. In the other three patients, tumor tissue was not present in the second biopsy. Continued precursor incorporation into DNA-synthesizing cells during treatment was detected in six of eight tumor specimens. In two tumor specimens, an increase in the percentage of S-phase cells was noted, in two specimens tumor cells synthesizing DNA were not detected, and in four specimens the percentage of S-phase tumor cells was lower than that in the pretherapy specimen. Patients in whom there were no S-phase cells detected during treatment or in whom no tumor was detected in the second biopsy had a favorable treatment outcome in comparison to those patients in whom continued tumor proliferation during treatment was detected. The number of cells in S-phase prior to the initiation of treatment was not predictive of whether or not proliferation would continue during cytotoxic therapy. Evidence for reentry of kinetically quiescent cells into the cycle during treatment was noted. Additionally, cytotoxic therapy altered the proliferation pattern of normal-appearing mucosa as well. The results of this study demonstrate that tumor cell proliferation does continue in some squamous cell carcinoma of the head and neck during intensive cytotoxic therapy.

INTRODUCTION

Malignant cell repopulation during cytotoxic therapy is believed to be a major impediment to the successful treatment of patients with rapidly proliferating malignant disease (1–5). This belief is based on a large body of indirect evidence, especially in the area of SCCHN (1, 6), which suggests that malignant cells continue to proliferate during radiotherapy, thereby offsetting, at least in part, the reduction in cell numbers produced by treatment. There is also indirect evidence that in some cases tumor proliferation rates increase during cytotoxic treatment, further compromising treatment outcome (7). Based primarily on the studies of experimental tumors (8–11) and on scant direct human data, new radiation therapy schedules have nevertheless been devised to overcome repopulation resistance (12). Additionally, indirect evidence has recently been presented that repopulation or regrowth resistance is also a significant impediment to successful treatment outcome in patients receiving chemotherapy (13).

Attempts to demonstrate the phenomenon of continued proliferation in patients during treatment using in vitro labeling with tritiated thymidine have been hampered by methodological problems. For example, in two studies investigators removed tumor tissue during treatment and labeled tumor fragments in vitro with [3H]thymidine to assess the effects of treatment on the proliferative rate (14, 15). Under these conditions, only a thin rim on the periphery of the tumor fragment incorporated the DNA label, thus limiting the information which can be obtained regarding the proliferative behavior of the tumor. Additionally, another group of investigators labeled tumor cell suspensions obtained by fine-needle aspiration, also relying upon in vitro uptake of [3H]thymidine (16). To study accelerated (or continuing) proliferation of tumor cells in patients, one must have the ability to measure proliferation parameters in vivo both before and during (or after) therapy. By administering IdUrd1 to patients before treatment and BrdUrd to patients after the treatment, in combination with immunohistochemical analyses of tumor biopsies obtained after each halogenated pyrimidine infusion, these measurements can be made (17).

In this article, we describe studies of the effects of cytotoxic therapy on SCCHN in vivo in patients. These studies have demonstrated that: (a) proliferation does continue unabated in some tumors during intensive cytotoxic therapy; (b) high pre-

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1 The abbreviations used are: IdUrd, iododeoxyuridine; BrdUrd, bromodeoxyuridine; 5-FU, 5-fluorouracil; CR, complete remission; PD, persistent disease; GMA, glycol methacrylate; ISEL, in situ end labeling; SCC, squamous cell carcinoma; SCCHN, squamous cell carcinoma of the head and neck.
therapy proliferative rates are not necessarily associated with continued proliferation during treatment; (c) some malignant cells which apparently leave the cell cycle shortly after the initiation of treatment resume proliferation 3–4 weeks later; and (d) the effects of cytotoxic therapy on the proliferative behavior of normal oral mucosa adjacent to a tumor can be markedly different from the effects on the tumor.

MATERIALS AND METHODS

Patient Characteristics and in Vivo Studies. Eleven squamous cell carcinomas of the head and neck were studied at \( t = 0 \) and 15–36 days later. The characteristics of the patients and their tumors are provided in Table 1. The patients received an infusion of IdUrd (100 mg/m\(^2\) over 1 h). At the end of the infusion, a biopsy of the tumor was obtained with care being taken to avoid necrotic areas. The biopsy specimen was immediately fixed as described below (\( t = 0 \) biopsy). The patients were then treated as described in Table 2. Six patients (patients 4, 5, 7, 8, 9, and 10) were treated with concurrent chemotherapy/radiotherapy in a week-on/week-off schedule. Chemotherapy consisted of: cisplatin, 60 mg/m\(^2\) i.v. bolus on day 1 and 5-FU, 800 mg/m\(^2\) continuous infusion on days 1–5 of weeks 1, 3, 5, 7, 9, 11, and 13. Radiation was given at 2 Gy/day on days 1–5 of weeks 1, 3, 5, 7, 9, 11, and 13. Each patient completed a 13-week course with a total irradiation dose of 70 Gy.

Patients 2 and 3 received alternating chemotherapy/radiotherapy (18). Chemotherapy consisted of: cisplatin, 20 mg/m\(^2\) i.v. bolus on days 1–4, and 5-FU, 200 mg/m\(^2\) i.v. bolus on days 1–5 of weeks 1, 4, 7, and 10. Radiation was given at 2 Gy/day on days 1–5 of weeks 2, 3, 5, 6, and at 1.5 Gy twice a day on days 1–5 of weeks 8 and 9. Total treatment duration was 10 weeks, with a total irradiation dose of 70 Gy. Patient 1 received one cycle of chemotherapy only (Taxol, 200 mg/m\(^2\) i.v. infusion on day 1, and cisplatin, 75 mg/m\(^2\) i.v. bolus on day 2). Patient 11 was treated with radiotherapy only (as above), and patient 6 received neutron beam therapy. Cytotoxic therapy began on days 3–5 after the first biopsy.

According to theoretical calculations, tumor cell repopulation may begin 3–6 weeks after the beginning of cytotoxic therapy (1). Based on these data, we chose to obtain the second biopsy (intratreatment biopsy) 15–36 days after the beginning of the cytotoxic therapy. Before the second biopsy, the patient received i.v. infusion of BrdUrd (100 mg/m\(^2\) over 1 h). With respect to the second biopsy, care was taken to biopsy nonnecrotic tissue at the site of the original tumor biopsy. In eight patients, tumor tissue was present in the second biopsy. Tumor tissue was not present in the second biopsy of the other three patients.

The results of treatment were assessed 6 weeks after the completion of the entire treatment course. At this time, the patients underwent endoscopic examination and computed tomographic scan. Patients without signs of tumor were categorized as being in CR. PD was characterized by gross tumor seen endoscopically or on computed tomographic scan 6 weeks after therapy.

Percentage of S-Phase Measurements. Detailed descriptions of the methods used in these studies can be found in previous publications (17, 19). In brief, the biopsy specimens were fixed and embedded in GMA or paraffin. Two-\( \mu \)-m-thin sections were prepared from the plastic-embedded sections, and 7-\( \mu \)-m sections were prepared from the tissues embedded in paraffin. The sections were rehydrated and treated with 3% \( \mathrm{H}_2\mathrm{O}_2 \), Pronase, and 4 N HCl. For specimens obtained after the infusion of IdUrd, monoclonal antibody 3D9 (a gift from Dr. G. L. Mayers, Roswell Park Memorial Institute, Buffalo, NY) was applied (1:200) for 30 min followed by biotinylated antimouse IgG (1:200) for 30 min, and then the ABC reagent is added (Vectastain Elite ABC kit). If the patient was infused with BrdUrd, tumor sections were stained according to the procedure described above using the rat anti-BrdUrd antibody (Sera Labs, Crawley Down) diluted 1:100 followed by staining with a Vectastain kit as described above. At the final step, all specimens were stained with 0.25% 3,3'-diaminobenzidine tetrahydrochloride in 0.003% \( \mathrm{H}_2\mathrm{O}_2 \) and counterstained with Harris’ hematoxylin solution. Sections treated without primary antibody were used as controls. In repeated staining sessions, it has been confirmed that the anti-BrdUrd monoclonal antibody is specific for BrdUrd and does not cross-react with IdUrd (19). In contrast, monoclonal antibody 3D9 recognizes both IdUrd and BrdUrd. In a previous study, 19 patients were infused with both IdUrd and BrdUrd, with a 30-min interval between infusions. Sections from these specimens were stained with either 3D9 or rat anti-BrdUrd monoclonal antibodies. The resulting labeling index values were very close to each other, indicating that both

<table>
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<tr>
<th>Patient</th>
<th>Age (yr)</th>
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<th>Differentiation</th>
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<th>TNM classification</th>
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<td>Tonsil</td>
<td>Moderate</td>
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* F, female; M, male.
halogenated pyrimidines are incorporated into DNA in the same manner (19).

The percentage of S-phase cells was determined in four to six randomly selected fields of the section. From 2000 to 5000 cells were counted in each section. In the second biopsies, where BrdUrd labeled cells were limited to some areas of the specimen, the percentage of S-phase values were calculated in these areas only. Special care was taken to distinguish between tumor tissue and dysplastic normal or interstitial tissue. Two experienced histopathologists read the slides independently, and consensus was reached on every specimen.

Some tumor cells in the second biopsy were the progeny of cells that were in the S-phase at the time of the IdUrd infusion just before the first biopsy. To be able to visualize these cells, label IdUrd/BrdUrd studies were available in only one half of the specimens. In the other four tumors, single labeling to detect BrdUrd was performed in paraffin-embedded specimens.

**Simultaneous Detection of S-Phase Cells and Apoptosis.** ISEL was carried out on GMA-embedded tumor (2 mm) sections as described in our previous report (20). Briefly, following pretreatment with sodium chloride-sodium citrate solution at 80°C and with 0.5% pepsin (Sigma, St. Louis, MO) in HCl (pH 2) at 37°C, the sections were incubated with a mixture of dATP, dCTP, and dGTP (0.01 m; Promega, Madison, WI), biotinylated dUTP (0.001 m; Sigma), and Escherichia coli DNA PolI (20 units/ml; Promega) at 18°C for 2 h. Incorporation of biotinylated dUTP was finally visualized using avidin-biotin-peroxidase conjugate (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) and diaminobenzidine. Thus, cells labeled positively for ISEL showed brown staining in their nuclei under light microscopy. Treatment of the tumor sections with monoclonal anti-IdUrd/BrdUrd antibody 3D9 to label cells in the S-phase was then carried out. Avidin-biotin-peroxidase conjugate (ABC kit) and diaminobenzidine were used to visualize ISEL labeling, and alkaline phosphatase-anti-alkaline phosphatase (DAKO, Carpinteria, CA) and nitroblue (Genius 1 kit; Promega) were used to visualize the S-phase cells. Thus, cells undergoing apoptosis (ISEL positive) exhibit brown staining in their nuclei, whereas the nuclei of the S-phase cells appear to be dark purple.

## RESULTS

**Effects of Treatment on Proliferative Rate.** Eleven patients with advanced SCC of the head and neck were studied before the initiation of treatment ($t = 0$ study) and on days 15–36 of treatment (intratreatment study). Of the latter, only eight had tumor tissue in the second biopsy. Six of these patients

<table>
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*CT, chemotherapy; RT, radiation therapy.*
Continued Tumor Cell Proliferation during Therapy

Cells labeled with IdUrd (C). Patient 3 had received an infusion of IdUrd before the first biopsy. Note numerous (33%) IdUrd-labeled cells in the first biopsy (A). The second biopsy, preceded by BrdUrd infusion (B), was obtained 22 days later, after two cycles of radiation (20 Gy) and chemotherapy (cisplatin + 5-FU). The tumor tissue contains a large number of S-phase BrdUrd-labeled cells (24%). Patient 4 received three cycles of radiation (30 Gy) and chemotherapy (cisplatin + 5-FU). Before the start of treatment, the tumor contained 19% S-phase cells labeled with IdUrd (C). Thirty-six days later most of the tissue at the primary tumor site was fibrotic. Small islands of tumor cells (D, arrow) survived with many cells synthesizing DNA, as demonstrated by in vivo BrdUrd labeling. A-D, X200.

Fig. 1  S-phase cells in biopsies obtained before and after treatment (paraffin, A and B; 3D9 (anti-IdUrd)/immunoperoxidase, C and D; anti-BrdUrd/immunoperoxidase staining, counterstained with hematoxylin). Patient 3 had received an infusion of IdUrd before the first biopsy. Note numerous (33%) IdUrd-labeled cells in the first biopsy (A). The second biopsy, preceded by BrdUrd infusion (B), was obtained 22 days later, after two cycles of radiation (20 Gy) and chemotherapy (cisplatin + 5-FU). The tumor tissue contains a large number of S-phase BrdUrd-labeled cells (24%). Patient 4 received three cycles of radiation (30 Gy) and chemotherapy (cisplatin + 5-FU). Before the start of treatment, the tumor contained 19% S-phase cells labeled with IdUrd (C). Thirty-six days later most of the tissue at the primary tumor site was fibrotic. Small islands of tumor cells (D, arrow) survived with many cells synthesizing DNA, as demonstrated by in vivo BrdUrd labeling. A-D, X200.

had newly diagnosed disease, and two had recurrent disease. Table 2 provides details regarding the treatment that each patient received, the relationship of the second proliferation study to the treatment being administered, and the response of the tumor to treatment. The intratreatment biopsy was obtained either in the last 1 or 2 days of cytotoxic therapy or within a few days of the completion of a course of therapy.

As can be seen in Fig. 1, A and C, prior to treatment the S-phase cells were distributed throughout the biopsy specimen. Treatment significantly disturbed the morphology of the tumor cells. In every treated tumor, the number of tumor cells was decreased. Many of the remaining cells were large with irregular shapes containing giant, often multilobulated nuclei. In seven of the eight tumors studied, the S-phase cells in the intratreatment biopsy were distributed throughout the tumor tissue (Fig. 1B). In one intratreatment specimen (Fig. 1D, patient 4), the proliferating tumor cells appeared to be organized into islands surrounded by fibrotic tissue. In every intratreatment biopsy, even in those that did not contain viable tumor tissue, there were many BrdUrd-labeled lymphocytes.

Table 3 provides data regarding the effects of cytotoxic therapy on tumor proliferation. In four specimens, the percentage of S-phase cells fell by 27, 37, 68, and 71% of the initial value (patients 3, 4, 5, and 6, respectively). In two specimens (patients 7 and 8), it fell to 0, and in two specimens, the intratreatment percentage of S-phase cells was greater than the pretherapy value (increases of 17% in patient 1 and 19% in patient 2). It is important to note that, as illustrated in Table 3, a high pretherapy percentage of S-phase cells was not an indication that a tumor was likely to continue to proliferate during cytotoxic therapy. In fact, a high pretherapy percentage of S-phase cells could be associated with a greater or lesser percentage of S-phase cells relative to the pretreatment value.

Five of six patients whose tumors contained proliferating cells in the second biopsy have died within 13 months after being studied, and one has relapsed. In contrast, two of three patients whose intratreatment biopsy did not contain tumor tissue are alive in complete remission (10+ and 27+ months, respectively), whereas the third patient died 14 months later from an unrelated cause. Similarly, one of two patients whose...
Evidence That the Same Cells in the Tumor Proliferate before and after Several Weeks of Treatment (Double Labeling of the Second Biopsies). In four of the second biopsies (four others were unavailable for this study for reasons described in “Materials and Methods”), double labeling demonstrated that many tumor cells contained IdUrd despite the 15–36-day hiatus between administration of the IdUrd and the second biopsy. In some of these cells (Table 4, patients 2 and 5), the intensity of staining for IdUrd was comparable to the intensity of staining of labeled cells immediately after the IdUrd infusion at \( t = 0 \) (Fig. 2A). In previous studies, we have found that the pyrimidine label becomes fragmented in cells that proliferate after being labeled with halogenated pyrimidines (19). Hence, the persistence of undiluted IdUrd label in the nuclei of tumor cells approximately 1 month after IdUrd administration strongly suggests that the IdUrd-containing cells in these tumors had undergone few, if any, divisions between the first and second biopsies.

Immediately after the intratreatment administration of BrdUrd, some tumor cells contained both IdUrd and BrdUrd (Fig. 2B). These double-labeled cells were in the S-phase during the pretreatment IdUrd infusion and were proliferating during the BrdUrd infusion as well, despite cytotoxic therapy. These double-labeled cells represented 4.6–19.1% of the tumor cell population (Table 4). In some of these cells, residual IdUrd label appeared to be undiluted and nonfragmented. The most likely explanation for this labeling pattern is that these cells stopped proliferating almost immediately after incorporating IdUrd and then, at approximately the time of the second biopsies, resumed proliferation.

In all tumors studied using the double-labeling methodology (Table 4), 8.5–18.4% of tumor cells contained fragmented IdUrd label. These cells had incorporated IdUrd during the first infusion before the start of the treatment and apparently had undergone one or more divisions since the IdUrd infusion. In one intratreatment biopsy (patient 7), after the administration of...
Day 0 biopsy: BrdUrd, all labeled cells contained fragmented IdUrd label only, and no tumor cells were labeled with BrdUrd (Fig. 3, A and B). As described below, although the cells of this tumor did not incorporate any BrdUrd at the time of the second labeling, adjacent normal mucosal cells incorporated BrdUrd. This result demonstrates that the pyrimidine was available and that the failure of tumor cells to incorporate BrdUrd is an indication that the tumor cells were not engaged in DNA synthesis.

The different types of labeled cells in biopsies taken during cytotoxic therapy are schematically represented in Fig. 4.

**Apoptosis during Cytotoxic Therapy.** Simultaneous cell cycle and apoptosis assessments were performed on the intratreatment biopsies of seven tumors. In none of the tumors were any S-phase cells undergoing apoptosis (ISEL positive), demonstrating that proliferating tumor cells were viable and not in the process of dying. In contrast, ISEL-positive cells were common in the fields of dying cells (Fig. 3C). In two specimens, a few double-labeled cells were detected (S-phase cells undergoing apoptosis) and were located in normal epithelium or in interstitial tissue.

**Changes in the Percentage of S-Phase Cells in Normal-appearing Mucosa during Treatment.** Normal mucosa in the second biopsy always contained the S-phase (BrdUrd-labeled) cells. Pretreatment and intrathepapy normal-appearing mucosa was present in four of the eight $t = 0$ and intratreatment biopsies. The percentage of the S-phase cells in the suprabasal region of normal mucosa was greater than that of the basal layer at $t = 0$ (Table 3). In contrast, in three of four specimens the percentage of S-phase cells in the basal layer of the intratreatment specimen was significantly greater than that of the $t = 0$ specimen (Fig. 5). In this small number of specimens, there appeared to be no consistent effect of cytotoxic therapy on the percentage of S-phase cells in the suprabasal layers of normal mucosa.

Double labeling of the second biopsy in three patients (patients 2, 5, and 7) shows that 25–36% of the labeled normal
mucosal cells contained both IdUrd and BrdUrd labels. This observation suggests that, as for tumor cells, normal mucosal cells which were in the S-phase before the initiation of treatment may either continue to divide during cytotoxic therapy or leave the S-phase to resume proliferation 3–4 weeks after the start of treatment.

Table 3 shows that there is no apparent relationship between the effects of treatment on the percentage of S-phase cells in the tumor and in adjacent mucosa. The independence of the S-phase behavior of the tumor and the adjacent normal mucosa is illustrated by the extreme example of the intratreatment specimen 7, in which the tumor cells failed to become labeled with BrdUrd whereas the normal tissue contained a high percentage of S-phase cells.

**DISCUSSION**

It is a central tenet of radiobiology that the continued proliferation of tumor cells during radiotherapy reduces the effectiveness of treatment. This phenomenon has been named repopulation resistance (3). The basis for the belief that repopulation during treatment compromises treatment outcome comes from indirect evidence: that the prolongation of a course of radiotherapy or interruptions in a course of radiotherapy reduce the likelihood of local disease control in SCCHN (1, 2). Additionally, clinical observations suggest that the rate of malignant cell proliferation may increase after 3–6 weeks of radiotherapy, further compromising treatment outcome (1).

The studies described here are the first to demonstrate that the tumors of some patients do indeed continue to proliferate during cytotoxic therapy. Although the number of patients studied to date is small, it is sufficient to establish the reality of repopulation during treatment. Additionally, the studies described here also demonstrate that some kinetically quiescent tumor cells reenter the cell cycle 2–6 weeks after the start of treatment, providing at least one explanation for the apparent acceleration of tumor proliferation postulated by others (1).

Moreover, in ISEL/BrdUrd double-labeled specimens, we did not see any tumor S-phase cells undergoing apoptosis. In other words, proliferating cells in our study did not belong to the population of dying cells. The absence of ISEL positivity among the S-phase cells in the SCCHN intratreatment specimens demonstrates that these cells are viable and are likely to be responsible for treatment failure in the future.

Of potential clinical importance is the observation that a high pretherapy proliferative rate is not necessarily associated with continued rapid proliferation during treatment (patients 5, 6, 7, and 8, Table 3). This observation suggests that some patients assigned to accelerated radiotherapy regimens on the basis of rapid pretherapy tumor proliferative rates may not require this type of therapy, a therapy associated with increased toxicity. The data in Tables 2 and 3 suggest that patients in whom the second tumor biopsy fails to demonstrate proliferating cells and patients in whom the second biopsy fails to reveal malignant tissue have a better prognosis than patients in whom the second biopsy demonstrates proliferating tumor cells.

The radiation tolerance of normal oral mucosa (which depends on cell proliferation rates among other factors) is an important aspect of cytotoxic therapy of SCCHN (21). The studies described here demonstrate that the rate of proliferation of the basal layers of normal mucosa, as predicted from animal studies in the literature (4), increases in response to cytotoxic therapy. In a larger series of normal mucosa samples, we have found that even though the basal layer of mucosa in untreated patients contained only 1.6 ± 0.2% S-phase cells, after cytotoxic therapy the value was significantly higher (19.0 ± 3.5%, P < 0.0001). In the suprabasal layers of treated patients, the frequency of S-phase cells (15.3 ± 2.6%) was significantly lower than that of untreated samples (31.6 ± 3.1%, P = 0.0002). The increase in the percentage of S-phase cells in the basal cell compartment after cytotoxic treatment is part of the proliferative response of stem cells to the damage inflicted by irradiation and chemotherapeutic agents. The discordance of the effects of cytotoxic therapy on the proliferative characteristics of the tumor and adjacent nontumorous mucosa in some patients demonstrates that treatment strategies designed to maximize the toxic effects of therapy on the tumor while minimizing effects on the adjacent normal mucosa will have to be based on knowledge of the response to treatment of these tissues in individual patients.

Our studies provide evidence that SCC tumor of the head and
neck can continue to proliferate both during the administration of cytotoxic therapy and between courses of therapy. Additionally, we have presented strong evidence that SCC cells at times become kinetically quiescent and are capable of resuming proliferation at a later time. The latter data are virtually identical to those described in the past for acute leukemia cells which were shown to become kinetically quiescent in vivo in patients and to resume proliferation in response to cytotoxic therapy (22). These observations, taken together with similar observations in acute myelogenous leukemia (23) and with the adverse prognosis associated with rapid pretherapy proliferative rates in both SCC and acute myeloid leukemia and in many other malignancies (24–29), strongly suggest that repopulation or regrowth resistance is a common phenomenon in malignant disease.

Finally, the development of strategies for reducing the regrowth of malignant cells, both during and between courses of cytotoxic therapy, would increase the therapeutic efficacy of currently available cytotoxic regimens (30). This approach would compliment the more common approach of dealing with resistant disease by increasing the intensity of cytotoxic therapy. We have already demonstrated that biological agents can slow the proliferation of malignant cells in vivo in patients (17).

REFERENCES
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