Synergistic Antitumor Effects of Topoisomerase Inhibitors and Natural Cell-mediated Cytotoxicity

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ABSTRACT

The mechanism of augmentation of tumor cell killing by immune effector cells and chemotherapeutic drugs was studied. The effect of treating tumor cells with various antineoplastic drugs on their sensitivity to murine natural cell-mediated cytotoxicity in vitro was investigated. Pretreatment with actinomycin D at nontoxic concentrations rendered L929 and WEHI-164 tumor cells more susceptible to killing by mouse spleen lymphocytes in a dose-dependent manner. Similarly, enhancement of L929 tumor cell killing by natural cell-mediated cytotoxicity was observed following treatment of the target cells with the topoisomerase II inhibitors, Adriamycin, amssacrine, bisantrene, etoposide, and teniposide, as well as with topoisomerase I inhibitor, camptothenic. In contrast, drugs which induce their cytotoxic effects by mechanisms that do not involve topoisomerase inhibition such as bleomycin, vinblastine, vincristine, and mitomycin C failed to exhibit synergism with natural cell-mediated cytotoxicity. However, moderate synergy was consistently observed with cis-platinum.

The effectors cells responsible for the cytotoxicity in the present system are natural cytotoxic cells since they kill WEHI-164 but not YAC cells, are resistant to treatment with anti-asialo-GM1 antibody, and their activity is abolished by anti-tumor necrosis factor antibodies. Indeed, tumor necrosis factor-mediated cytotoxicity of WEHI-164 or L929 was enhanced by treatment of the target cells with topoisomerase II inhibitors. Moreover, WEHI-164 cells selected for tumor necrosis factor resistance were resistant to natural cell-mediated cytotoxicity, and no synergy could be observed with topoisomerase inhibitors.

INTRODUCTION

Recent advances in cancer immunotherapy using recombinant cytokines (TNF, colony-stimulating factor, interleukin 2, and interferon) and immune cells (lymphokine-activated killer cells and T-lymphocytes) render it possible to rationally design and implement adjunct immunochemotherapy strategies for cancer treatment. Furthermore, the better understanding of the molecular mechanisms involved in the destruction of tumor cells by immune effector cells and chemotherapeutic drugs would facilitate the selection of drug combinations and protocols which will result in effective destruction of the tumor cells. Synergy between cytotoxic immune cells and antineoplastic drugs is well known (1–4). For example, certain tumor cells that are resistant to cytolyis by lymphocytes or macrophages become sensitive to killing following treatment with actinomycin D (1–3). Furthermore, the sensitivity of tumor cells to cytotoxic products of immune effector cells such as TNF and lymphotoxin is enhanced following treatment with actinomycin D (5, 6). The exact mechanism of this synergy is not fully elucidated. However, recent studies have demonstrated that topoisomerase II inhibitors, including actinomycin D, synergize with TNF in killing tumor cells sensitive to this cytotoxicity (7, 8).

In the present report, we investigated the potential synergistic effect of chemotherapeutic drugs with different mechanisms of action on natural cell-mediated cytotoxicity against tumor cells and attempted to define the drug and effector cell specificity in such a synergy. We demonstrated that treatment with topoisomerase I or II inhibitors augments the sensitivity of tumor cells to killing by normal mouse spleen cells. Drugs that act via mechanisms other than topoisomerase inhibition had weak or no effect. Furthermore, the synergy was most potent with effector cells defined previously as NC (9–12) but not with NK cells.

MATERIALS AND METHODS

Animals. Specific pathogen-free C3H/HeN mice were obtained from Charles River Breeding Laboratories (Kingston, MA). The mice were used for experiments at 6–12 weeks of age.

Drugs. The following chemotherapeutic drugs were used in this study: Act D, ADR, BL, CIS, MMC, VB, and VCR were purchased from Sigma Chemical Co., St. Louis, MO. mAMSA, BIS, CPT, VP16, and VM26 were kindly provided by Drug Synthesis and Design and Natural Products Branches of the National Cancer Institute, Bethesda, MD. TNF (2 × 10^6 units/ml protein) was provided by J. Chan, Smith Kline & French Laboratories, King of Prussia, PA.

Target Cells. The following murine tumor cell lines were used: L929 fibrosarcoma; WEHI-164, a chemically induced fibrosarcoma of BALB/c mice; YAC-1, a lymphoma induced in A strain mice by Moloney murine leukemia virus, and UV-2237, a fibrosarcoma induced in a C3H mouse by chronic UV irradiation. WEHI-164 TNF-R is a TNF-resistant variant of WEHI-164 established by repeated culturing with TNF-contained medium. L929 and UV-2237 were maintained in CMEM at 37°C in a humidified atmosphere of 5% CO_2 in air. The other cell lines were maintained in RPMI 1640. All cell lines were routinely examined for and were found to be free of Mycoplasma infection.

Effector Cells. Mice were killed by cervical dislocation and the spleens were removed. A single cell suspension was prepared by mechanically disrupting the spleens and forcing them through a 60-mesh wire screen. Erythrocytes in the spleen cell preparations were removed by water lysis. In some experiments, nylon wool-nonadherent spleen cells were prepared as described previously (13).

Treatment with Chemotherapeutic Drugs. The fibrosarcomas were harvested by treatment with 0.25% trypsin and 0.02% EDTA solution and were washed once with CMEM. Lymphoma cells grown in suspension were collected and washed before use. Cells (2.5 × 10^7/ml) were mixed with various concentrations of drugs in polypropylene tubes and were incubated for 3 h with occasional shaking in 5% CO_2 at 37°C. At the end of the incubation period, the cells were washed with CMEM to remove drugs and then labeled with ^31Cr for the cytotoxicity assay.

Antibody-Complement Treatment. Nylon wool-nonadherent spleen cells (5 × 10^7/ml) were treated with anti-asialo-GM1 antibody (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at final dilution of 1:50 for 45 min at 4°C. The cells were then washed and incubated with rabbit complement at 1:15 dilution (Cedarlane Laboratory, Ltd., Hornby, Ontario, Canada) for 45 min at 37°C. The cells were washed and adjusted to 1 × 10^7/ml before use in the cytotoxicity assay.
Cytotoxicity Assay. Mouse spleen cell-mediated cytotoxicity was determined by 51Cr release assay at 4 h for YAC-1, 14 h for WEHI-164, and 18 h for L929 and UV-2237 as described previously (14). Briefly, tumor cells were labeled with 200 µCi of 51Cr (Na2CrO4) for 60 min at 37°C with occasional shaking and, after washing three times, cells were used as targets. Effector cells were prepared as a single cell suspension as described above. In the assay, a graded number of effector cells were mixed with 51Cr-labeled target cells in a total volume of 200 µl medium per well of 96-well round-bottomed microtiter plates (Costar) and then incubated at 37°C for 4 or 18 h in 5% CO2 in air. After centrifugation, 100-µl aliquots of supernatant were harvested and the amount of 51Cr released into the supernatants was measured in a gamma counter. The percentage of killing was calculated according to the following formula:

\[
\% \text{ of cytotoxicity} = \frac{T - S}{M - S} \times 100
\]

in which \(T\) is cpm released in the presence of effector cells, \(S\) is cpm released from target cells incubated without effector cells, and \(M\) is cpm released from target cells added with 0.1 N NaOH.

Statistical Analysis. The Student t test (2-tailed) was used. All data presented are means of triplicate measurements in representative experiments out of more than three independent experiments.

RESULTS

To determine the range of drug doses to be used for the synergy experiments with immune cells, we examined the direct effect of drugs against 51Cr-labeled L929 cells in a 24-h chromium release assay (Fig. 1). As expected, L929 cells exhibited different degrees of sensitivity to the various chemotherapeutic drugs used. Based on these results, drug concentrations that exhibit little or no direct cytotoxicity were used for further studies. These suboptimal concentrations were as follows: Act D, 0.1–5 µM; ADR, 5–50 µM; mAMSA, 0.5–5 µM; BIS, 0.5–2.5 µM; VP16, 5–25 µM; VM26, 0.5–2.5 µM; CPT, 1–10 µM; BL, 0.02–0.1 µM; CIS, 5–25 µM; MMC, 5–25 µM; VB, 1–10 µM; and VCR, 2–10 µM.

Based on previous studies demonstrating enhancement of macrophage-mediated killing by Act D, we examined whether pretreatment with Act D renders L929 fibrosarcoma cells more sensitive to killing by normal mouse spleen cells. L929 cells were incubated with 0–5 µM Act D for 3 h at 37°C; then the cells were washed and labeled with 51Cr before addition to the effector cells. This treatment protocol was selected after preliminary experiments showed that the drugs do not affect 51Cr incorporation or release and that their efficacy is equal to that observed after continued exposure for the 24 h of the cytotoxicity assay. The results shown in Fig. 2 demonstrate that treatment with 0.5, 1.0, or 5.0 µM Act D rendered L929 cells more susceptible to CMC as compared to untreated control target cells. Spontaneous 51Cr release from L929 cells treated with 0.1–1.0 µM Act D in the absence of effector cells did not differ from controls (26–28% release). A small increase, however, was observed in cells treated with 5.0 µM drug where spontaneous release was 6% above control value.

The specificity of the synergy with immune effector cells was examined by treating the L929 cells with various chemotherapeutic drugs before testing their sensitivity to CMC. Since Act D may inhibit DNA topoisomerase I (15), we evaluated the effect of topoisomerase II inhibitors (ADR, VP16), topoisomerase I inhibitors (CPT), and VB which does not act via topoisomerase inhibition. The results shown in Fig. 3 demonstrate that significant enhancement of CMC was observed with to...
poisomerase I and II inhibitors but not with VB. This profile was further confirmed when additional antineoplastic drugs with different mechanisms of action were used (Table 1). Treatment with Act D, ADR, mAMSA, BIS, VP16, and VM26, all of which are DNA poisomerase II inhibitors, enhanced the cytotoxicity of spleen cells against L929 by 116, 80, 43, 36, 42, and 42%, respectively. Also, treatment with CPT enhanced the CMC against L929 cells by 80%. In contrast, drugs which exert their cytotoxic effect by mechanisms that do not involve poisomerase inhibition such as BL, MMC, VB, and VCR had little or no effect on CMC against this target cell. CIS was the exception as it consistently enhanced CMC against L929 cells.

Having demonstrated that drug specificity exists for synergy with normal immune effector mechanisms against one tumor target cell, the applicability of this observation to other cell lines was investigated. This is of importance since different tumor cells exhibit differential sensitivity to killing by natural effector cells which include macrophages, NK or NC cells. For this purpose, WEHI-164 fibrosarcoma, UV-2237 fibrosarcoma, and YAC-1 lymphoma cells were used. The direct cytotoxic effect of the drugs against each target cell was determined and subtoxic concentrations were used for the synergy experiments.

The results shown in Table 2 demonstrate that tumor cell treatment with Act D and VP16 enhanced spleen cell-mediated killing of L929 and WEHI-164 cells but failed to affect the killing of UV-2237 and YAC cells. Considering that the latter target cells are killed primarily by NK cells, whereas the former ones exhibit high sensitivity to NC cell killing, it appears that the synergy between antineoplastic drugs and immune effector cells is influenced also by the nature of the effector mechanisms involved in the destruction of the target cells.

To further define the type of effector cells involved in the destruction of L929 and WEHI-164 cells, spleen cells were passed over a nylon wool column to remove adherent macrophages, then treated with anti-asialo-GM1 antibodies and complement to eliminate NK cells. Such treatment reduced the destruction of L929 and WEHI-164 cells, while the activity against YAC cells was totally abolished (Table 3). Using this effector cell preparation, we were able to demonstrate enhanced killing of Act D-treated L929 or WEHI-164 cells as compared to cells not treated with the drug. These data indicate that the effector cells responsible for the synergistic effects observed are NC and not NK cells.

Recently, it was reported that the cytotoxic effects of NC cells are mediated through the secretion of TNF (16, 17). Therefore, the potential synergy between TNF and poisomerase II inhibitors in killing L929, WEHI-164, and YAC-1 cells was examined. As shown in Table 4, significant enhancement of TNF-mediated killing of L929 and WEHI-164 was observed following treatment with Act D. Because of the high sensitivity of WEHI-164 cells to TNF, synergistic effects were maximally attained at lower TNF concentrations (10 and 100 units/ml). In contrast, the low levels of TNF-mediated cytotoxicity observed with YAC cells were not enhanced following treatment with Act D. Similar results were obtained by using other poisomerase II inhibitors (data not shown).

To examine whether the induction of TNF resistance affects the sensitivity to killing by NC cells or the synergy observed with poisomerase II inhibitors, TNF-resistant WEHI-164 cells were established by repeated culturing in escalating concentrations of TNF. The results shown in Table 5 indicate that, unlike the parent WEHI-164 cells, the TNF-resistant cells were completely resistant to killing by mouse spleen cells, a response which could not be affected or enhanced by treatment with either Act D or VP16.

**DISCUSSION**

In this study we investigated the synergistic activities between antineoplastic drugs and host immune effector mechanisms in tumor cell destruction and whether this synergy is restricted to a class of drugs with a common mechanism of action and/or to a subpopulation of effector cells. The results demonstrate that treatment with DNA poisomerase I or II inhibitors augmented the sensitivity of L929 tumor cells to killing by normal spleen cells. Most chemotherapeutic drugs which exert their cytotoxic effect by mechanisms other than poisomerase inhibition exhibited weak or no effect. The synergy observed with cis-platinum may be explained by the ability of this drug to produce protein-concealed DNA strand breaks.

Schlager (4) reported that ADR-treated P815 murine mast-cytoma cells were more sensitive to killing by antibody-depend-
ent cytotoxicity and MMC-treated P815 cells showed enhanced sensitivity to killing by cytotoxic T-cells. In this study, the mechanism of augmentation apparently involved a change in the physical properties of the cell membrane. Other investigators (1-3, 18) have demonstrated that preincubation with Act D induced selective sensitivity of WEHI-164 to monocytomediated killing. Additionally, of the different chemotherapeutic drugs tested, ADR and cyclohexamide had an enhancing effect; whereas VCR, 5-fluorouracil, methotrexate, and 1,3-bis(2-chloroethyl)-1-nitroso-urea were ineffective (18). In this study, using different tumor target and effector cells, we demonstrated that the sensitivity to killing by normal spleen lymphocytes is enhanced primarily following treatment of the tumor target cells by topoisomerase inhibitors. The results obtained with Act D, ADR, and VCR are consistent with those of Coletta et al. (18). It should be emphasized, however, that this observation does not apply to all tumor cells evaluated nor to all effector immune mechanisms. For example, UV-2237 fibrosarcoma and YAC-1 lymphoma cells failed to show enhanced sensitivity to NCMC after treatment with topoisomerase inhibitors. In contrast, WEHI-164 cells behaved in a fashion similar to that of L929 cells. This difference, however, may be dependent on the nature of the effector cells involved in tumor cell killing, as WEHI-164 and L929 are killed primarily by natural cytotoxic cells (16, 17) (Table 2); whereas YAC-1 and UV-2237 tumor cells are sensitive to NK cell-mediated cytotoxicity (14, 17) (Table 3).
Natural cytotoxic cells are present in mouse spleens and human peripheral blood and exhibit spontaneous cytotoxicity similar to that described for NK cells (9). The two cells, however, differ by surface antigenic markers, kinetics of tumor cell lysis, and tumor cell specificity (9–11). Moreover, the mechanism of NC- but not NK-mediated killing involves the secretion of TNF (16, 17). Indeed, treatment with anti-TNF antibodies abolished NCMC against WEHI-164 and L929 and no synergy with Act D could be observed. In contrast, treatment with anti-asialo-GM1, only marginally reduced NCMC and failed to affect the synergy with Act D.

The possibility that the synergistic activity between NC cells and topoisomerase inhibitors is mediated by enhanced tumor target cell sensitivity to TNF-mediated cytotoxicity is supported by the following evidence. (a) Induction of TNF resistance renders WEHI-164 cells resistant to NC-mediated killing and to synergy with chemotherapeutic drugs; (b) synergy was observed between Act D and TNF in killing L929 and WEHI-164 cells but not YAC-1 and UV-2237 cells which are relatively resistant to TNF-mediated cytotoxicity; and (c) drug specificity for synergy with TNF has been demonstrated (7, 8) to be similar to that described for NC cells in the present study.

The mechanisms by which topoisomerase inhibitors render tumor cells susceptible to immune effector mechanisms, such as NC cells and TNF, have not been fully elucidated. The effects, however, are most likely related to an increase in the level of topoisomerase-mediated DNA damage. Recent studies have demonstrated that DNA fragmentation is induced by TNF, lymphotoxin, and cytotoxic T-lymphocytes, and that DNA damage is a key process in the cytotoxicity exhibited by these immune effector mechanisms (20, 21). Therefore, it would not be surprising to find synergy between topoisomerase inhibitors and NC cells or TNF since DNA cleavage, albeit induced via different molecular mechanisms, constitutes, a central event leading to cell death in both cases. This is further supported by the observation that synergy with topoisomerase inhibitors was not observed with tumor cells selected for resistance to killing by NC cells or TNF. The possibility of a direct drug-mediated activation of the effector cells (22) could be ruled out as being responsible for the enhanced cytotoxicity, since pretreatment of the target cells with topoisomerase inhibitors followed by extensive washing was as effective as maintaining the drugs throughout the incubation with the effector cells. This observation, together with results from experiments measuring cellular uptake of radiolabeled drug, rule out the possibility that enhanced drug uptake by the tumor target cells is responsible for the enhanced tumor cell destruction observed in the present system.

The clinical relevance of the synergy between selected chemotherapeutic drugs and host defense mechanisms remains to a large extent unknown. In experimental models, however, evidence has been provided in support of synergistic antitumor effects between chemotherapeutic drugs and host immunity (21). Similarly, using murine bladder, tumor enhancement of the antitumor efficacy of the topoisomerase inhibitors actinomycin D and etoposide was obtained in vivo following combination therapy with TNF (8).

Together, our data and previously published reports may provide a basis for a rational selection of chemotherapeutic drugs and immunomodulators that when used in combination therapy would synergize for tumor destruction in vivo.

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