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# The Specific Cytotoxic Effects of Daunomycin Conjugated to Antitumor Antibodies

**Ronald Levy,[1] Esther Hurwitz, Ruth Maron, Ruth Arnon, and Michael Sela**

*Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel*

## SUMMARY

Daunomycin was covalently bound to immunoglobulins by periodate oxidation as described in the preceding paper. Conjugates were prepared with immunoglobulins directed against either of two mouse lymphoid tumors or with nonspecific immunoglobulins. These conjugates were tested for their toxic effects on various tumor target cells as measured either by their inhibition of RNA synthesis or by their reduction of the growth of the tumor cells after transplantation. We found that the drug preferentially affected the target cells that the antibody to which it was attached could recognize. These daunomycin-antibody conjugates are therefore sufficiently toxic and selective in their effects to be potentially useful in *in vivo* therapeutic studies.

## INTRODUCTION

There has been increasing interest recently in attempting to bind antitumor drugs to antibodies in order to enhance the effectiveness of these agents against tumor cells and to reduce their toxicity for normal cells. Antimetabolites (11), toxins (12, 13), enzymes (16), and alkylating agents (1, 3, 5, 6) have been linked to antibodies with variable degrees of success in improving the selectivity of their cytotoxic effects. In the preceding report (8), we investigated the binding of adriamycin and daunomycin to immunoglobulins by several covalent methods. Conditions were found whereby these drugs could be linked to antibodies, preserving a significant degree of drug activity as well as antibody activity. In this paper we have prepared a series of antibody-daunomycin conjugates and have studied their toxicity against several different tumors *in vitro*. We have found that the effects of these drugs can be directed against specific target cells by binding them to the appropriate antibodies.

## MATERIALS AND METHODS

The sources of all chemicals and reagents were those described in the preceding paper (8).

**Tumors.** Several murine lymphoid tumors were used in this study. These include a carcinogen-induced B-cell leukemia in SJL/J mice (7), a Moloney virus-induced lymphoma (YAC) in A/J mice (10), and a mineral oil-induced plasmacytoma (PC5) in BALB/c mice (17). In addition a lymphoma induced in Lewis rats by the intrathymic injection of murine radiation leukemia virus was also used. This rat lymphoma shares viral-related cell surface antigens with the PC5 plasmacytoma but not with the other mouse tumors used here (M. Haas, unpublished observation). All tumors were maintained by passage in their respective inbred animal strains.

**Antisera.** Antiserum to BSA[2] (anti-BSA) was produced in rabbits by weekly s.c. injection of 2 mg BSA emulsified in complete Freund's adjuvant.

Rabbit antisera to the B leukemia cells and to the PC5 cells were prepared by 4 to 5 i.v. injections of $10^8$ tumor cells at 5-day intervals. Antibody activity was measured by complement-dependent cytotoxicity as previously described (8). Titers of 1:100 to 1:200 were obtained for these antisera against their respective immunizing cells. The anti-B leukemia antisera showed similar cytotoxicity against the YAC tumor cells. These antisera also reacted against normal SJL/J mouse spleen cells. The reactivity against normal spleen cells could be removed by absorption, but when this was performed the antisera were much less potent against the tumor cells. Therefore, for the experiments reported here, the anti-B leukemia antisera were used in the unabsorbed form. The anti-PC5 antisera were used after absorption with normal BALB/c thymus and spleen cells and in the absorbed form were cytotoxic against both the immunizing PC5 cells and the rat lymphoma cells, but not against the YAC cells.

The immunoglobulin fractions of these antisera were prepared by precipitation with ammonium sulfate at 33% saturation and used for the preparation of drug conjugates.

**Daunomycin-Immunoglobulin Conjugates.** Daunomycin was covalently bound to immunoglobulins by periodate oxidation of the drug and reaction of the oxidized drug with the proteins, followed by borohydride reduction of the addition products, as described previously (8). These conjugates contained from 2 to 10 moles of daunomycin per mole of protein.

**Pharmacological Effects of the Drug Conjugates.** The specific cytotoxicity of drug-immunoglobulin conjugates

[1] Fellow of the Helen Hay Whitney Foundation. Permanent address: Department of Medical Oncology, Stanford Medical School, Stanford, Calif.

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[2] The abbreviation used is: BSA, bovine serum albumin.

was tested after allowing them to attach to target cells during a short incubation *in vitro*, washing them to remove nonspecific proteins and their drug conjugates, and examining the cells for residual drug effects. Tumor cells were washed and suspended in Eagle's medium at a concentration of $2 \times 10^7$ cells/ml and dispensed into the wells of microtiter plates (Dynatec Laboratories, Sussex, England) in 50-$\mu$l volumes. Various concentrations of free drug, immunoglobulins, or drug-immunoglobulin conjugates were added in 50-$\mu$l volumes and, after agitation (Dynatec AM 69 microshaker), the plates were incubated for 5 min at 37°. One hundred $\mu$l of medium were then added to each well, and the plates were centrifuged at 4° at 1800 rpm (International PR-J centrifuge equipped with microtiter plate carriers) for 10 min. Supernatants were removed by a single shake of the inverted plates, and the wells were refilled with 200 $\mu$l of fresh medium. This washing procedure was repeated 1 more time, and the cells were finally resuspended in Eagle's medium, 100 $\mu$l/well, and incubated for 2 hr at 37° in a humidified atmosphere of 5% $CO_2$ in air. Ten $\mu$l containing 1 $\mu$Ci of [$^3$H]uridine were then added to each well and, after a further 1 hr incubation, 25 $\mu$l of 25% trichloroacetic acid were added. Trichloroacetic acid precipitates were washed, solubilized in NaOH, and counted for radioactivity as previously described (19). The results are expressed as percentage of inhibition of [$^3$H]uridine incorporation, compared to the control which contained either 0.9% NaCl solution or free antibody at a concentration equivalent to the antibody concentration of the corresponding conjugate. The variation of triplicates in this assay was generally less than 10%.

In addition to the [$^3$H]uridine incorporation assay, the target tumor cells were tested for their ability to grow after transplantation. After exposure to the drug conjugates *in vitro* and washing, the cells were transplanted into their respective syngeneic strains, and the survival of recipients was followed.

## RESULTS

**Specific Cytotoxicity of Daunomycin Anti-B Leukemia Conjugates.** Daunomycin was conjugated to both anti-B leukemia and anti-BSA immunoglobulins and tested for cytotoxicity against the B leukemia cells as well as several other tumors *in vitro*. As shown in the preceding report (8), these conjugates retained approximately 50% of the activity of the free drug. The different tumors used here had similar sensitivities to the drug-immunoglobulin conjugates. For the present experiments a concentration of daunomycin-immunoglobulin conjugate was used that gave 40 to 60% inhibition of [$^3$H]uridine incorporation in test cells when it was left in contact with the target cells for the entire period of the incubation. To reveal the specificity of the conjugates, the test cells were exposed to them for only 5 min, to allow attachment of specific antibody and then washed to remove nonspecific immunoglobulins, and the toxicity of the daunomycin remaining in contact with the cells was assessed as described in "Materials and Methods."

From the results shown in Table 1, it can be seen that the daunomycin-anti-B leukemia conjugate showed significant residual inhibition of [$^3$H]uridine incorporation in the B leukemia cells after this brief exposure and washing. When different target cells were tested in this assay, we found that their sensitivity to the specific daunomycin-anti-B leukemia conjugate followed the specificity of the antibody. That is, this conjugate was toxic to the cross-reacting YAC cells but not to the noncross-reacting PC5 or rat lymphoma cells (Table 1, Line 1), although the sensitivity of these cells to the free drug was, if anything, greater than that of the B leukemia cells (Table 1, Line 4). The specific effect observed here depended upon the antibody activity since no effect was seen with the daunomycin-anti-BSA conjugate (Table 1, Line 2). In the case of the B leukemia test cells, the effect of the daunomycin-antibody conjugate was even greater than that of the free drug (Table 1, Line 4, Column 2). Free antibody did not render the cell more sensitive to the effects of the free drug (data not shown) but increased slightly the effect of the nonspecific daunomycin-anti-BSA conjugate on these cells (Table 1, Line 3, Column 2). The heavy agglutination of the cells caused by the antibody may have resulted in some trapping of the daunomycin-anti-BSA, conjugate, making it more difficult to remove by washing. YAC cells, which were not strongly agglutinated by the anti-B leukemia antibodies, were not affected by the mixture of anti-B leukemia and daunomycin-anti-BSA (Table 1, Line 3, Column 3).

In addition to their effects on RNA synthesis, the conjugates were tested for their effects on tumor cell growth. After brief exposure of the B leukemia cells *in vitro* to the conjugates, free antibodies, or free drug, the cells were washed and transplanted in the syngeneic SJL/J host. Chart 1 shows the survival curves of animals receiving $10^7$ cells each. The mean survival of animals receiving untreated cells was 11 days. None of the other control groups, which received cells exposed to free drug, free antibody, or the mixture of anti-B leukemia antibody and

Table 1

*Specific cytotoxicity of daunomycin linked to anti-B leukemia*

Drug (0.6 $\mu$g) either as the protein conjugate or free drug was incubated with $10^6$ cells in a total volume of 100 $\mu$l for 5 min at 37°. Medium was then removed, and cells were washed and resuspended in fresh medium and pulsed with [$^3$H]uridine at the end of 2 hr of further incubation.

| Incubated with | % inhibition of [$^3$H]uridine incorporation | | | |
|---|---|---|---|---|
| | B leukemia | YAC | PC5 | Rat lymphoma |
| Daunomycin-anti B leukemia | 38[a] | 42[a] | 17[b] | 9[b] |
| Daunomycin-anti-BSA | 1[b] | 0[b] | 4 | 9 |
| Daunomycin-anti-BSA anti-B leukemia | 18[c] | 0[d] | ND[e] | ND |
| Free daunomycin | 17 | 49 | 33 | 41 |

[a, b, c, d] Difference between Value *a* and Value *b*: $p < 0.001$ by the Student *t* test; difference between Value *a* and Value *c*: $p < 0.05$; difference between Value *a* and Value *d*: $p < 0.001$.

[e] ND, not done.

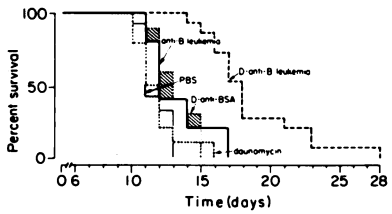


Chart 1. B leukemia cells were exposed for 5 min *in vitro* to 0.15 M NaCl:0.01 M phosphate (pH 7.2) (*PBS*), free daunomycin, anti-B leukemia, daunomycin-anti-BSA (*D-anti-BSA*), or daunomycin-anti-B leukemia (*D-anti-B leukemia*). Cells were then washed and transplanted into syngeneic SJL/J mice, $10^7$ cells/animal. Drug and protein concentrations were the same as those used in Table 1.

daunomycin-anti-BSA, differed in their survival from those receiving untreated cells. Only the group receiving cells exposed to the specific daunomycin-anti-B leukemia conjugate showed prolonged survival. Their survival was equivalent to that of animals receiving only $10^3$ untreated cells.

**Specific Cytotoxic Effects of Daunomycin-Anti-PC5 Conjugates.** Similar experiments were performed using daunomycin conjugated to anti-PC5 immunoglobulins. Once again it can be seen (Table 2) that the specific conjugate showed toxicity against the homologous PC5 target cell and against the cross-reacting rat lymphoma cells, but much less against the noncross-reacting YAC cells. In this series of experiments the daunomycin-anti-B leukemia conjugate was included as a specificity control, showing the converse pattern of toxic effects. Again, the homologous system, daunomycin-anti-PC5 against the PC5 test cell, was even superior to the effect of free drug on the same cells.

When the growth of the treated PC5 cells was examined in syngeneic BALB/c animals, we found a slight effect of free drug as well as of free antibody, but the specific daunomycin-anti-PC5 conjugates resulted in an even greater effect, including failure of tumor take in 50% of the animals (Chart 2). The survival of this group of animals was equivalent to that of animals receiving only $10^2$ untreated tumor cells. The long-term survivors were resistant to subsequent challenge with $10^3$ tumor cells.

In an additional series of experiments, the cells were treated briefly *in vitro* as before but transplanted without washing, relying on dissipation of drug and nonspecific drug

Table 2

*Specific cytotoxicity of daunomycin linked to anti-PC-5 immunoglobulin*

Drug (1.5 μg) was used; other conditions were identical to those in Table 1.

| | % inhibition of [$^3$H]uridine incorporation | | |
|---|---|---|---|
| Incubated with | PC5 | Rat lymphoma | YAC |
| Daunomycin-anti-PC5 | 60[a] | 63[a] | 20[b] |
| Daunomycin-anti-BSA | 7[b] | 14[b] | ND[d] |
| Daunomycin-anti B leukemia | 16[b] | 14[b] | 62[a] |
| Free daunomycin | 32[c] | 53 | 67 |

[a, b, c] All *a* values different from all *b* values by $p < 0.001$ (Student's *t* test; *a* values different from *c* values by $p < 0.001$.

[d] ND, not done.

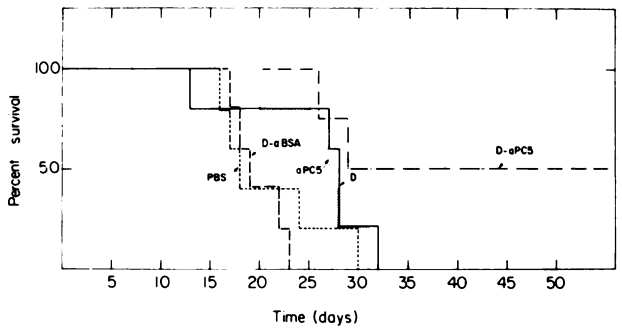


Chart 2. PC5 cells were exposed for 5 min *in vitro* to 0.15 M NaCl:0.01 M phosphate (pH 7.2) (*PBS*), daunomycin-anti-BSA (*D-aBSA*), anti-PC5 (*a-PC5*), free daunomycin (*D*), or daunomycin-anti-PC5 (*D-aPC5*). Cells were then washed and transplanted into syngeneic BALB/c mice, $10^7$ cells/animal. Drug and protein concentrations were the same as those used in Table 2.

conjugates in the animal. The results, shown in Chart 3, are similar to those in Chart 2. An additional control group is shown in which cells were exposed to a mixture of free drug and anti-PC5 antibody, which resulted in no apparent effect over that of either the free drug or the free antibody alone.

## DISCUSSION

In the present series of experiments, we have shown that daunomycin covalently bound to antibodies directed against individual tumors showed preferential cytotoxicity against these specific tumor cells. When the drug was bound to anti-B leukemia antibodies, the conjugates were toxic to the homologous B leukemia cells as well as to the cross-reacting YAC cells (Table 1) but were not significantly toxic to the noncross-reacting PC5 or rat lymphoma cells (Tables 1 and 2). Conversely, when the drug was conjugated to anti-PC5 antibodies, they were toxic to PC5 cells and cross-reacting rat lymphoma cells but much less toxic to YAC cells (Table 2). Therefore, the property of target cell recognition can be imparted to daunomycin by attaching it to the appropriate antibody. For the experiments described here, it was convenient to use different tumor cells as targets, since their sensitivities to the free drugs were similar, their growth after treatment was easily followed in the transplanted host, and antibodies of differential specificity were readily obtained.

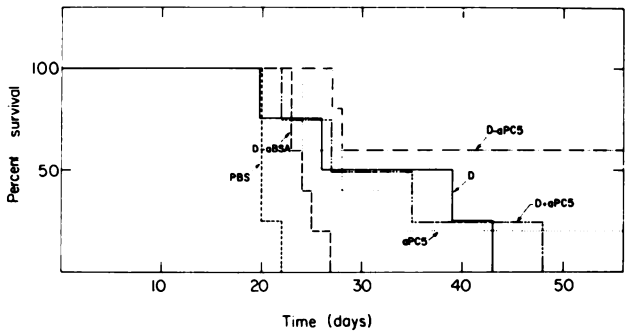


Chart 3. Same conditions and abbreviations as in Chart 2, except that cells were transplanted after the *in vitro* treatment without washing.

The present experiments were additionally simplified in that target cells were exposed to drug-antibody conjugates *in vitro*, avoiding all of the problems of antibody localization of tumor cells *in vivo* (2, 9, 18). However, once specific attachment of these conjugates to target cells was allowed to occur *in vitro*, the subsequent growth of the tumor cells *in vivo* was retarded (Chart 1) and, in the PC5 system, sometimes prevented entirely (Chart 2). No toxicity was detectable during the brief time of exposure to the conjugates *in vitro*, even by the sensitive [$^3$H]uridine incorporation assay. Therefore, these results indicate that the attachment of the drug-antibody conjugate to the cells must have persisted *in vivo* for at least the 2 to 3-hr period needed to express their cytotoxic effects. This persistent attachment was due to antibody activity, since exposure to drug conjugated to unrelated immunoglobulins caused no retardation of tumor growth (Chart 2) even when the cells were not washed to remove these nonspecific conjugates prior to transplantation (Chart 3).

Several other groups of investigators have linked cytotoxic agents to antitumor antibodies. Moolten *et al.* (12, 13) have covalently bound diphtheria toxin to antidinitrophenyl antibodies or to antibodies against mumps virus and have studied the cytotoxicity of these conjugates in a model system in which target cells contained either dinitrophenyl or mumps antigens on their surface. The most notable characteristic of their system is the extremely high potency of the toxic agent (15).

Philpott *et al.* (16) have used a different approach, linking the enzyme glucose oxidase to antitrinitrophenyl antibodies. In the presence of this conjugate, lactoperoxidase, glucose, and sodium iodide, tumor cells bearing trinitrophenyl determinants on their surface were preferentially iodinated, leading to their reduced survival in tissue culture. This system has the advantage that the toxicity of the conjugate is expressed on the cell membrane, and it does not need to enter the cell to have its effect. It is, nevertheless, a complex system, requiring the carrier antibody, 2 enzymes, and 2 substrates, which may prove to be cumbersome to use *in vivo*.

Several investigators have used the alkylating drug chlorambucil, combining it in a noncovalent, stable-to-dialysis complex with antitumor antibodies (1, 3, 5, 6). The experiments of Rubins and Dulbecco (20) showed that the mixture of drug and free antibody was as effective as the drug-antibody complex in preventing the growth of BHK tissue culture cells. It is possible, therefore, that noncovalent complexes of antibody and chlorambucil dissociate *in vivo* and act independently in a synergistic fashion. One possible mechanism for this synergism has been provided by the recent experiments of Segerling *et al.* (21), who showed that the susceptibility of cells to lysis by antibody and complement can be greatly enhanced by their prior exposure to inhibitors of RNA or protein synthesis.

In the present studies we have chosen to work with the antitumor antibiotics adriamycin and daunomycin, which have an extremely wide spectrum of antitumor activity and an acceptable level of normal tissue tolerance (4, 14). The finding that these drugs can be covalently bound to proteins with retention of their drug activity raises the possibility that the effectiveness of these agents can even be improved. Attaching these drugs to the appropriate antibodies enabled them to express a selective cytotoxic effect *in vitro*. As methods are developed for the isolation and purification of tumor-specific antibodies, it should eventually be possible to test the potential of antibody-directed chemotherapy *in vivo* using adriamycin and daunomycin.

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