Superiority of an Acid-labile Daunorubicin-Monoclonal Antibody Immunoconjugate Compared to Free Drug

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ABSTRACT

We conjugated the chemotherapy agent daunorubicin to the anti-T-cell monoclonal antibody T101 using an active ester intermediate of the acid-labile linker cis-aconitate anhydride. By converting carbohydrate hydroxyl groups on the antibody to amines prior to conjugation, average drug to antibody ratios of 25:1 were achieved with retention of cytotoxicity and only minimal loss of immunoreactivity. The pH sensitivity of the linkage was confirmed. The preparation was cytotoxic for antigen-bearing cells but not antigen-negative cells, even up to 48-h incubation in vitro. Specific cytotoxicity was apparently mediated through the endocytosis of cells but not antigen-negative cells, even up to 48-h incubation in vitro. and only minimal loss of immunoreactivity. The pH sensitivity of the drug to antibody ratios of 25:1 were achieved with retention of cytotoxicity. Slow metabolism and/or excretion would be desirable in order to prolong duration of drug exposure and opportunity for binding to tumor cells. Ideally, such conjugates would have minimal immunogenicity so that therapy could be given repeatedly.

INTRODUCTION

MoAbs1 are potentially useful in cancer therapy in association with either complement or effector cells, as regulators of cell proliferation or as passive immunization (1, 2). Alternatively, MoAbs may be used as carriers of cytotoxic agents such as radioisotopes, natural toxins, or chemotherapy drugs (1, 3). Durable antitumor effects have been seen in occasional patients treated with antibody alone (4, 5), but in most trials, only limited tumor regressions have been observed (6–10). MoAbs may be useful as carriers of chemotherapy agents to improve therapeutic index by increasing drug uptake by tumor cells, by decreasing drug toxicity on normal cells, and by prolonging the bioavailability of the drug for more extensive exposure to tumor cells. An ideal conjugate would carry many molecules of drug per molecule of antibody, but retain immunoreactivity while binding selectively and specifically to tumor cells. Ideally, it would be nontoxic in the conjugated form, but would release the drug preferentially in or near tumor and thereby retain cytotoxicity. Slow metabolism and/or excretion would be desirable in order to prolong duration of drug exposure and opportunity for binding to tumor cells. Ideally, such conjugates would have minimal immunogenicity so that therapy could be given repeatedly.

In the past, we conducted clinical trials with murine MoAb T101 in patients with chronic lymphocytic leukemia and cutaneous T-cell lymphoma (9, 11–13). Limited antitumor effects were observed, perhaps due to the limited cytotoxic potential of T101, and modulation of the CDS antigen via internalization (14, 15). However, such internalization could be an advantage for cytotoxic T101 immunoconjugates. In an effort to assess this, we developed a tumor model using human MOLT-4 cells as s.c. xenografts in athymic mice (16). We demonstrated persistence of the human CDS antigen in these xenografts, and confirmed that the kinetics of antigenic modulation were similar to those previously observed in vitro and in vivo (16). We previously reported results obtained with a noncovalent doxorubicin-T101 complex (17), a T101-doxorubicin-dextran immunoconjugate (17), a T101-methotrexate conjugate (18), and T101-rinA chain immunotoxin (19) using in vitro DNA and protein inhibition assays and our in vivo tumor model. In this manuscript, we describe generation of an acid-labile immunoconjugate consisting of T101 and the chemistry agent DNR and its preclinical testing in these same in vitro and in vivo systems.

MATERIALS AND METHODS

Preparation of cis-Aconitate-Daunorubicin. Preparation of CA-DNR was performed using a modification of the method of Shen and Ryser (20) as depicted in Fig. 1. Cis-acronic anhydride (Sigma Chemical Company, St. Louis, MO) was dissolved in dioxane (Aldrich, Milwaukee, WI) at room temperature and then added drop-wise, while stirring, to an ice-cold solution of DNR (Ives Laboratories, New York, NY) which had been dissolved in 0.5 ml of 0.1 M Na2HPO4 at pH 8.5. The reaction mixture was stirred at 0°C for 15 min and then at room temperature for 15 min. Then the reaction mixture was again placed on ice for 1 h while HCI was slowly added with stirring until a heavy precipitate formed. The CA-DNR precipitate was collected by centrifugation at 4°C, dried, and redissolved in 50 mM DMF (Aldrich) and the pH was adjusted to 8. Thin-layer chromatography was used to confirm that free DNR was not present in the final preparation.

Formation of an Active Ester of cis-Aconitate-Daunorubicin. At this point, 100 mM N-hydroxysuccinimide (Aldrich) was coupled to 50 mM CA-DNR in the presence of anhydrous DMF and 100 mM dicyclohexylcarbodiimide (Aldrich). This reaction mixture was stirred at room temperature for 1 h and then incubated overnight at 4°C in the dark to form an active ester intermediate. This reaction chemically favors esterification of the γ-carboxyl group of cis-aconitate (20). Analysis by thin-layer chromatography confirmed a broad band of ester moving with the solvent front. This was collected and stored in the dark at –70°C.

Derivatization of Antibody. T101 (Hybritech, San Diego, CA) is an IgG1 murine MoAb that binds the CDS antigen on malignant and normal T-lymphocytes, thymocytes, and CLL cells (11). Hydroxyl groups on T101 were oxidized to aldehydes using 0.02 M sodium perperiodate at pH 4 at 4°C. Because of antibody structure, this oxidation mostly involves carbohydrate groups located predominantly on the constant region of the MoAb heavy chain. Satisfactory formation of an active ester of the CDS antigen was confirmed spectrophotometrically by reaction with N-(2,4-dinitrophenyl)-β-alanylglycylglycine hydrazide as detailed elsewhere (21). After passage through a Sepharose CL-6B column equilibrated with 0.05 M acetate buffer (pH 6.5), oxidized antibody was incubated with a 200- to 400-fold molar excess of ethylenediamine for 1 h at room temperature which results in creation of additional amino groups by reaction with the free aldehydes. The reducing agent sodium cyanoborohydride (Aldrich) was added to a final concentration of 5–10 mM to stabilize this reaction. The solution was incubated for an additional 4 h and then dialyzed at 4°C against PBS to remove excess ethylenediamine and sodium cyanoborohydride.

Received 5/6/88; revised 7/29/88; accepted 8/4/88.

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1 This work was supported by Biotherapeutics, Inc., National Cancer Institute Contract N01-CM-47672, and the Veterans Administration.
2 To whom requests for reprints should be addressed, at Scripps Clinic and Research Foundation (MS-312), 10666 N. Torrey Pines Road, La Jolla, CA 92037.
3 The abbreviations used are: MoAbs, monoclonal antibodies; DNR, daunorubicin; CA-DNR, cis-aconitate-daunorubicin; DMF, dimethyl formamide; IC, immunoconjugate; [3H]DNR, tritiated daunorubicin; PBS, phosphate-buffered saline; cpm, counts per minute.
Immunoconjugate Formation. The activated CA-DNR in DMF was added to the derivatized T101 in PBS with constant stirring, and then incubated for 18 h at room temperature in the dark. This resulted in production of the immunoconjugate by binding of the esterified carboxyl groups to the amino groups on the derivatized MoAb (Fig. 1). The IC was separated from free CA-DNR by desalting on a Sephadex G25 column (Pharmacia, Piscataway, NJ) in PBS. Further purification steps to exclude noncovalently bound DNR included dialysis against PBS, and use of a Sephadex G100 sizing column (Pharmacia). The molar ratio of DNR:T101 was determined by measuring DNR absorbance at 495 nm with an extinction coefficient of 81%/cm = 218 and by assaying protein concentration using the Bradford protein method. In various lots, drug:MoAb ratios of 10–40:1 were obtained with an average of 25:1. For the experiments described below, ratios were 28–32:1.

Cell Lines. For in vitro surface binding and cytotoxicity assays, MOLT-4, 8402, and 8392 cell lines were maintained as continuous cell suspensions in RPMI 1640 medium with 10% fetal calf serum (Irvine Scientific, Irvine, CA). MOLT-4 and 8402 are human T-cell leukemia lines with which T101 reacts and 8392 is the B-cell autologous lymphoblastoid counterpart to 8402; 8392 cells do not express CD3. Cells in log phase with viabilities exceeding 90% by trypan blue exclusion were used in the assays.

Immunoreactivity. Indirect immunofluorescence assays were used to demonstrate binding of T101 or T101-CA-DNR to various cells. Non-specific mouse IgG2a RPC5 [Litton Bionetics, Frederick MD] was used as a negative control. Aliquots of 10^6 cells were incubated for 30 min at 4°C with a test reagent, washed twice, and then incubated another 30 min with fluorescein-conjugated, affinity-purified goat antimouse F(ab')_2 (Boehringer Mannheim, Indianapolis, IN). Serial dilutions of T101 were compared to serial dilutions of T101-CA-DNR based on T101 equivalencies. We did not test the immunoreactivity of the oxidized and ethylenediamine-substituted T101 (i.e., without DNR) intermediates. Fluorescence analysis was performed on the Ortho Cytofluorograf 500 with 2100-H computer connection (Ortho, Westwood, MA).

Acid Lability of T101-CA-DNR. Various samples of T101-CA-DNR and T101-CA-[^3]H]DNR ([^3]H]DNR, Amersham, Chicago, IL) were incubated at 37°C at pHs 3–8 for 1, 2, 4, 24, or 48 h. Suspensions were then passed through a Sephadex G25 column to separate free drug from drug bound to antibody. Absorption at 495 nm, ^3[H]-cpm, and protein concentration were measured to determine how much DNR had been released from T101 at each pH and point in time.

Fate of [^3]H]DNR during Immunoconjugate-induced Modulation. 10^6 MOLT-4 cells were incubated with [^3]H]DNR-T101 for 30 min at 4°C to allow binding but inhibit antigenic modulation and internalization. Cells were then washed, cpm redetermined, and washed again prior to incubating in PBS at 37°C for 0, 1, 2, 4, and 24 h. At each time point cells were washed and the combination of PBS supernatant and wash was considered the extracellular fraction. The cells were then sonicated for 10 min at 1,000 × g. The supernatant was removed and the nuclear pellet counted. The supernatant was centrifuged for 60 min at 33,000 × g and the lysosome-associated pellet counted. Residual supernatant was sampled for membrane and ribosome-associated counts.

Cell Growth Inhibition Assays. The incorporation of [^3]H]thymidine (Amersham) was used to measure cell growth inhibition of MOLT-4, 8402, and 8392 cells (17). The inhibiting effect of T101 alone, T101 + DNR, free DNR, and the T101-DNR IC was compared. Triplicate cultures were grown in 96-well flat-bottom microtiter plates (Costar, Van Nuys, CA). Aliquots of 4 × 10^3 cells in 100 µl of culture media were mixed with 100 µl of various concentrations of IC or drug. Reagents and cells were incubated at 37°C for 4 h and 48 h. At that time 0.5 µCi of [^3]H]thymidine was added to each well and cells were incubated at 37°C for 18 h and were then harvested onto glass fiber filter paper using a cell harvester. Filter paper was dried, placed in a vial with scintillation fluid, and counted in a scintillation counter.

Animal Tumor Studies. In vivo animal tumor studies were carried out in BALB/c (nu/nu) athymic mice bearing palpable, measurable s.c. tumors of the MOLT-4 cell line as we have previously described (16–18). All animals had palpable, measurable tumors of about 3 x 3 mm or 9 mm^2 at the time therapy was started. Each individual experiment contained six animals in each of five arms including a PBS control, T101 alone at a dose of 500 µg, DNR alone at a dose of 30–40 µg, the IC at a dose containing 500 µg T101 and 30–40 µg DNR, and a combination of 500 µg T101 mixed with 30–40 µg DNR in the same syringe. Two different IC lots were used in these experiments containing 30 and 40 µg of DNR. Two sets of animal experiments were performed with each preparation.

Each animal received a single i.p. injection. Animals were observed daily for general well being and survival. Maximum cross-sectional...
RESULTS

Immunoreactivity. Indirect immunofluorescent staining comparing T101-CA-DNR binding and T101 binding on MOLT-4 T-cells (CD5+) and 8392 B-cells (CD5−) showed that T101-CA-DNR was specific for cells expressing CD5 (Table 1). Non-specific binding to B-cells was not detected. For serial dilutions of the IC and free T101, no difference could be detected between the two antibody preparations until concentrations were decreased to 2.5 to 1.0 μg/ml. At that point, a decrease in immunoreactivity of the IC was apparent.

Acid Lability of the Daunorubicin Immunoconjugate. The pH sensitivity of the IC is shown in Fig. 2. The data shown is for absorbance and similar results were obtained when [3H]DNR release was determined. Drug release was pH dependent with almost 100% of DNR released at pH 3 and 4, after 2 to 4 h of incubation. No detectable DNR was found at pH 7–8; roughly 50% of DNR was released at pH 5. This released DNR retained cytotoxic activity in vitro.

Cellular Fate of the Immunoconjugate. The intracellular localization of [3H]DNR after binding to target cells during and following antigenic modulation is illustrated in Fig. 3. During the first hour of incubation, membrane counts dramatically decreased while nuclear and lysosomal intracellular counts increased consistent with internalization of [3H]DNR. Roughly, equivalent cpm were present in the nuclear and lysosomal fractions at each time point. There was also some egress off the membrane and/or efflux of [3H]DNR into the extracellular fraction following incubation at 37°C. Similar experiments with [125I]T101 showed similar internalization of antibody followed by an efflux of some immunoreactive T101 (24).

In Vitro Cytotoxicity. The inhibition of [3H]thymidine uptake associated with various concentrations of T101, free DNR, T101 + DNR, or T101-CA-DNR for MOLT-4 target cells and 8392 controls following incubation for 48 h is shown in Fig. 4. Results for a 4-h incubation yielded similar results and thereby confirmed specificity and cytotoxicity of the IC. The IC was as effective as both free DNR and T101 + DNR against MOLT-4 cells. Free DNR was equally effective over a similar dose range for both MOLT-4 and 8392 cells (i.e., no specificity), while the IC was as ineffective as T101 alone against the CD5− 8392 cells. The combination of T101 + DNR lacked specificity in vitro. The retention of specificity up to 48 h is additional evidence for the lack of significant contamination of the IC by noncovalently bound DNR. We previously demonstrated that noncovalently bound anthracycline, such as DNR or doxorubicin, can provide some specific cell killing in short term culture, and even in vivo in an animal tumor model (17).

Animal Tumor Studies. The tumor growth curves for both mean and median tumor sizes animals with established MOLT-4 tumors are illustrated in Figs. 5 and 6. There were 24 animals in each arm from four different experiments. The growths of PBS- and T101-treated tumors were similar to those seen in other T101 IC experiments (17–19). The growth curves for the
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Fig. 4. Specific inhibition of \(^{3}H\)thymidine uptake by 8392 B-cell (A) as opposed to MOLT-4 T-cells (B) in a 48-h assay in the presence of various molar concentrations of T101 (C), DNR alone (D), and equivalent concentrations in a T101-CA-DNR immunoconjugate (E), and T101 + DNR (F). Concentration of T101 was 500 \(\mu\)g. The concentration of DNR was 30–40 \(\mu\)g.

Fig. 5. Growth curves of MOLT-4 tumors growing in athymic mice expressed as the median value from the tumor cross-sectional diameter products at each point in time, illustrating superiority of the T101-CA-DNR immunoconjugate. The medians of 24 tumors were plotted for each time point in each treatment arm. All tumors were 5–9 mm in size when therapy was initiated. Included are single i.p. injections of PBS as a control (G), T101 alone (H), T101 + DNR (I), and T101-CA-DNR immunoconjugate (J).

Fig. 6. Superiority of the T101-CA-DNR immunoconjugate as evidenced by growth curves of MOLT-4 tumors growing in athymic mice expressed as the means of tumor cross-sectional diameter products at each point in time with standard error. The means for 24 tumors were plotted for each time point in each treatment arm. All tumors were 5–9 mm in size when therapy was initiated. Included were single i.p. injections of PBS as a control (K), T101 alone (L), DNR alone (M), T101 + DNR (N), and T101-CA-DNR immunoconjugate (O). Concentration of T101 was 500 \(\mu\)g. The concentration of DNR was 30–40 \(\mu\)g.

Table 2 T101-CA-DNR immunoconjugate therapy in nude mouse MOLT-4 tumor model: summary of therapeutic efficacy following a single injection

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of mice</th>
<th>Partial</th>
<th>Complete</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>T101</td>
<td>24</td>
<td>2 (8%)</td>
<td>1 (8%)</td>
<td>3 (12%)</td>
</tr>
<tr>
<td>DNR</td>
<td>24</td>
<td>3 (12%)</td>
<td>1 (8%)</td>
<td>4 (17%)</td>
</tr>
<tr>
<td>T101 + DNR</td>
<td>24</td>
<td>3 (12%)</td>
<td>2 (17%)</td>
<td>5 (21%)</td>
</tr>
<tr>
<td>T101-CA-DNR</td>
<td>24</td>
<td>4 (17%)</td>
<td>14 (58%)</td>
<td>18 (75%)</td>
</tr>
</tbody>
</table>

The actual regressions in each treatment arm are shown in Table 2. The IC resulted in a response rate of 75% which was superior to each of the other arms (overall, \(\chi^2 = 43.11, df = 5, P < 0.0001\)). Experiments using T101-CA-DNR with 30–40 \(\mu\)g DNR produced more responses than earlier IC with 12 or 16 \(\mu\)g DNR (18) (18/24 versus 1/18, \(P < 0.001\) by Fisher’s exact test). In a subsequent series of experiments, the T101-CA-DNR was clearly superior to other CA-MoAb conjugates consisting of DNR and antibodies that had no, or limited, activity with the MOLT-4 line. In addition, T101-DNR MoAb IC had no activity \(in\, vivo\) against melanoma tumors in athymic mice, while an antimelanoma-CA-DNR conjugate gave excellent suppression as a positive control (Table 3).

DISCUSSION

We previously demonstrated the efficacy of a noncovalently linked T101-doxorubicin IC (17), a T101-methotrexate IC (18), and a T101 ricin A-chain immunotoxin (19) compared to various controls in this same nude mouse model. In the present study, we used an active ester of cis-aconitate-linked DNR and a derivatized MoAb to increase amino binding sites in order to produce an acid-labile T101-CA-DNR-IC with drug:MoAb ratios of 28–32:1. The derivatization allowed us to approximately double the amount of drug bound to MoAb, which is theoreti-
An animals received a single i.p. injection with murine MoAbs containing equivalent amounts of DNR. T101 does not react with the M21 cell line while the antimelanoma MoAb does. The experiment was terminated after 21 days; and there were six animals in each arm.

<table>
<thead>
<tr>
<th>Day</th>
<th>Control</th>
<th>MEL-CA-DNR</th>
<th>T101-CA-DNR</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>18.0</td>
<td>18.0</td>
<td>19.7</td>
</tr>
<tr>
<td>4</td>
<td>45.2</td>
<td>21.7</td>
<td>52.5</td>
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<td>7</td>
<td>63.7</td>
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</tr>
<tr>
<td>14</td>
<td>108.2</td>
<td>24.3</td>
<td>119.7</td>
</tr>
<tr>
<td>18</td>
<td>149.2</td>
<td>34.2</td>
<td>161.8</td>
</tr>
<tr>
<td>21</td>
<td>211.3</td>
<td>37.7</td>
<td>195.8</td>
</tr>
</tbody>
</table>

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Table 3 Lack of antitumor effect for T101-CA-DNR in nude mouse M-21 melanoma model

In summary, our results indicate that use of an active ester of a pH-sensitive linker and DNR can be combined to a derivatized MoAb using site-directed methods to enhance amine binding sites, to produce a satisfactory IC. This IC retains reasonable immunoreactivity, releases free DNR under acid conditions, is selectively cytotoxic to antigen-bearing cells, and produces superior in vivo results compared to various controls in an animal tumor model system. This approach merits further investigation and may be applicable for human clinical trials.

ACKNOWLEDGMENTS

We wish to thank Susan Wormsley of Cytometrics for the cytofluorographic analysis performed. We wish to thank Kathleen Meyers and Jo Ann Meyer for their assistance in the preparation of the manuscript.

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