Anthracycline Immunoconjugates Prepared by a Site-specific Linkage via an Amino-Dextran Intermediate Carrier

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ABSTRACT

Anthracycline, either daunomycin or doxorubicin, was site specifically attached to the carbohydrate moiety of a monoclonal anticarcinoembryonic antigen antibody by using amino-dextran as the intermediate carrier. The reaction resulted in an immunoconjugate that contains approximately 20 to 25 molecules of drug per molecule of immunoglobulin G. Flow-cytometric studies revealed the retention of the antibody-binding activity. The immunoconjugate was cytotoxic to the target cells, as examined by the ³⁵Selenomethionine incorporation studies, and remained efficient for targeting a human colon cancer (GW-39) in the nude mouse model. The conjugate possessed a greater antitumor activity against the subcutaneous tumor than either the free drug or an irrelevant antibody conjugate, and it was well tolerated by the animals at a much higher dose level than was the unconjugated drug.

INTRODUCTION

The major problem of cancer therapy is the inadequacy of the treatment to distinguish between normal and tumor cells. A general lack of discriminatory power for the currently utilized treatment procedures necessitates maintaining a balance between the tumoricidal effects and host toxicity. The maximal tolerated dose that patients can receive, whether determined by bone marrow toxicity or other major organ dysfunctions, in the majority of cases is lower than is necessary to destroy the tumor. Antibodies that react with their target antigen in a specific manner potentially can be utilized to increase the maximal tolerated dose by increasing the specific delivery of the cytotoxic agents to the tumor. Cytotoxic agents that would normally be too toxic to the patients when used in a systemic fashion may be coupled to the antibodies in such a manner that the toxic actions are now directed more to the cells that express the target antigen than to normal tissues (1-6).

In our laboratory, a site-specific linking method (7, 8) was developed by which the cytotoxic agents are linked to the carbohydrate moiety of antibody via a bridging polymer (amino-dextran with a molecular weight of 40,000), resulting in an immunoconjugate with a high substitution ratio, retention of immunoreactivity, preferred tumor-targeting properties, and an improved antitumor activity in tumor xenografts (8, 9). It is our desire to extend this conjugation method to anticancer agents with different modes of action for use in combination therapy. In this study, antibodies of the anthracycline family, such as daunorubicin and doxorubicin, were conjugated at the carbohydrate moiety of monoclonal antibodies that react with CEA, resulting in immunoconjugates with a high substitution level as well as preferred immunological properties. The chemical modifications, tumor targeting, and inhibitory effects of these immunoconjugates on cultured cells and on tumor xenografts are the subject of this study. Due to the similarity of the conjugation methodology between doxorubicin and daunomycin, the results of the DOX-antibody conjugate are the focus of this paper.

MATERIALS AND METHODS

Reagents. Reagent-grade daunomycin, doxorubicin, and ECDI were purchased from Sigma Chemical Co. (St. Louis, MO). The injection grade of doxorubicin was kindly provided by Cetus Corp. (Emeryville, CA). Other chemicals were obtained from Aldrich (Milwaukee, WI).

Target Cells. LoVo, a human colon cancer cell line, was obtained from American Type Culture Collection (Rockville, MD) and was maintained in culture in Dulbecco's modified Eagle's medium that was supplemented with 5% heat-inactivated fetal bovine serum, 5% equine serum, penicillin (100 μg/ml), streptomycin (100 μg/ml), and glutamine (2 mm). The cells were routinely passed after detachment with 0.5% trypsin/0.2% EDTA (Gibco, Grand Island, NY).

Antibodies and Tumor Model. NP-4, an anticarcinoembryonic antigen murine monoclonal antibody IgG1, was purified from mouse ascites by Protein A affinity chromatography and was kindly supplied by Immunomedics, Inc. (Newark, NJ). The specificity of this antibody and its tumor targeting in GW-39 tumor-bearing animals (10, 11), as well as the clinical evaluation of this antibody, have been described (12). The mouse ascites of the irrelevant antibody, anti-AFP (IgG1), was generously provided by Immunomedics, Inc., and was purified on a Protein A-MAPS II affinity gel (Bio-Rad, Richmond, CA). This antibody has limited affinity on the target cell (LoVo) and was therefore used as a control antibody to construct the irrelevant immunoconjugate.

GW-39, a human colon carcinoma, was initially established in the cheek pouch of the golden hamster (13) and was also serially maintained in nude mice (Harlan Sprague-Dawley, Indianapolis, IN). The tumor was transplanted s.c. as a 10% tumor suspension (w/v) in PBS in the presence of gentamicin. A volume of 0.2 ml of the cell suspension was injected per animal. Tumors averaging 0.1 to 0.2 g are generally obtained 2 wk after transplantation.

Amino-Dextran and the Preparation of the Drug-Dextran Intermediate Conjugates. Amino-dextran, with an average molecular weight of 40,000, was prepared by the same procedure that was described previously (7, 8). The procedures for the preparation of DOX- or DNR-dextran were similar, except for the reaction conditions (Fig. 1). Briefly, either DOX or DNR, at a 10-mg quantity (17 μmol), was dissolved in 0.6 ml of anhydrous dimethylformamide. To this solution, 7.5 μl of triethylamine were added, followed by 1.9 mg of succinic anhydride. The mixture was reacted at room temperature for 18 h (the reaction for DNR can be conducted at 50°C because of its stability). ECDI (35 mg in 0.4 ml of water) was added. After 50 min at room temperature, 15.8 mg of amino-dextran in 0.8 ml of distilled water were then introduced, and the mixture was reacted at room temperature for 48 h. The mixture was then filtered to remove unreacted amino-dextran. The immunoconjugate was purified by Protein A affinity chromatography and was stored at -20°C.

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3 The abbreviations used are: CEA, carcinoembryonic antigen; PBS, phosphate-buffered saline (0.01 M phosphate:0.15 M NaCl, pH 7.2); DNR, daunomycin; DOX, doxorubicin; ECDI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; PGA, polyglutamic acid; FUR, fluorouridine; IgG, immunoglobulin G; AFP, alpha-fetoprotein; HPLC, high-pressure liquid chromatography; IC₅₀, dose causing a 50% inhibition of protein synthesis; LD₅₀, 50% lethal dose (LD₅₀ defined similarly).
SITE-SPECIFIC CONJUGATION OF ANTHRACYCLINES

Fig. 1. Synthetic scheme for the preparation of DOX and DNR immunoconjugates.

for an additional 5 h. The mixture was then purified on a Sephadex G-25 column that had been equilibrated with water. The drug-dextran peak was collected and lyophilized. The ratio of drug substitutions per dextran molecule was determined by absorbance at 482 nm for the drug concentration (ε = 154 for 1% DOX, ε = 139 for 1% DNR in PBS) and by the dry weight of dextran.

Preparation of the Drug-Dextran-Antibody via Site-Specific Attachment. The method of site-specific attachment of drug-dextran to the carbohydrate moiety of antibody has been described (7, 8). In this preparation, the procedure was slightly modified so that the antibody concentration during the oxidation was 10 mg/ml of PBS, pH 5.5. After separation on Sephadex G-25, the antibody was concentrated to 10 mg/ml and reacted with 2 equivalents of drug-dextran for 24 h. After the Schiff base was reduced by NaBH₃CN, the conjugates were purified by extensive dialysis (Spectrum cellulose dialysis tubing; Mₜ cutoff, 50,000) until no detectable drug-dextran was present in the filtrates. A dialysis procedure was chosen because of the high nonspecific affinity of the drug (or drug-dextran) to either the gel filtration matrix or Amicon filtration membrane. The purity of the conjugates was examined by HPLC on a Zorbax GF-250 gel filtration column (DuPont, Wilmington, DE) to ensure the absence of the intermediate molecular weight, or small molecular weight materials was calculated and compared with that of the original conjugate.

Immunoreactivity of the Conjugates. The immunoreactivity of the conjugates was examined by flow cytometry of the target cells. Briefly, 1 × 10⁶ LoVo cells were incubated with different concentrations of antibody or conjugate in a final volume of 100 μl for 60 min. After washing, the surface-bound antibody (or conjugate) was detected by fluorescein-labeled goat anti-mouse antibodies. The fluorescence intensity and the percentage of positive binding were measured on a FACScan flow cytometer (Becton Dickinson, Braintree, MA).

Tumor Targeting of the Conjugates in the GW-39/Nude Mouse Model. The conjugates were labeled with ¹³¹I by the chloramine-T procedure (15). After labeling, the integrity of the conjugates was examined on a CEA-affinity column to determine the immunoreactivity and on a HPLC gel filtration column equipped with an in-line radioactivity detector (Beckman, Fullerton, CA) to determine radiopurity. The labeled conjugate (25 μCi) was then coinjected with 10 μCi of ¹²⁵I-labeled free antibody into the GW-39 tumor-bearing nude mice (female, 6 to 8 wk old) via tail vein injection. Groups of 5 animals were sacrificed at 24, 72, and 168 h after injection. Tumor and major organs were removed for radioactivity determination. After the correction of the down-scattering effect and physical decay, the radioactivity associated with the tissues was expressed as the percentage of injected dose per gram. The targeting efficiency of the conjugates was compared directly to the unconjugated antibody.

In Vitro Cytotoxicity of Conjugates. The cytotoxic activity of the conjugates was examined on LoVo cells by following the ⁷⁵selenomethionine incorporation into the cells. Briefly, the cells were trypsinized and suspended in RPMI 1640 medium supplemented with fetal bovine serum (5%), equine serum (5%), glutamine (2 mm), penicillin (100 units/ml), and streptomycin (100 μg/ml) to a concentration of 3 × 10⁵ cells/ml. One hundred μl of the cell suspension were added
per well to a 96-well polystyrene microtiter plate (Dynatech, Chantilly, VA). The cells were allowed to attach and were then treated with different concentrations of the conjugate (freshly dialyzed to remove trace amounts of DOX or DOX-dextran that may have released from the conjugate upon storage), as well as the controls, for 24 h. 

77Selenomethionine (0.1 μCi/well) was added, and the cells were incubated for an additional 18 h for protein incorporation. The cells were then harvested by using a semiautomatic cell harvester (Skatron, Sterling, VA). The radioactivity associated with the cells was counted in a Packard γ-scintillation spectrometer and compared with that of control medium. The cytotoxic activity of the conjugates was calculated according to the following equation and expressed as the percentage of inhibition of 77selenomethionine incorporation,

\[
\% \text{ of inhibition} = 1 - \frac{(\text{cpm}_{\text{control}} - \text{cpm}_{\text{cyclodecimide}})}{(\text{cpm}_{\text{control}} - \text{cpm}_{\text{cyclodecimide}})} \times 100.
\]

Cycloheximide at 50 μM was used as the positive-killing control. The radioactivity associated with the cells that were treated with this agent was considered as the baseline and was subtracted from the other treatment groups.

Antitumor Activity of Conjugates in Tumor Xenografts. Antitumor efficacy of the conjugates was evaluated in nude mice bearing GW-39 tumors. The tumor was transplanted as described above. After the tumor was established (approximately 8 to 9 days after the transplantation and when the tumor was about 8 x 8 x 5 mm), the animals were divided randomly into groups of 6, and they were given the conjugates at different dose levels by twice weekly i.p. injections for a total of 7 injections. The tumors were measured in 3 dimensions with a hand caliper and were calculated as \( \frac{1}{2} \times \text{length} \times \text{width} \times \text{depth} \) in cm\(^3\). The calculated tumor volumes closely correlated with the actual tumor weight. Host toxicity was followed by body weight loss. The animals were sacrificed on Day 24 after the initiation of the treatment, and the tumors were removed for morphological evaluation to confirm the therapeutic effects. Various controls that were included in this study were the free drugs, the intermediate conjugates (drug-dextran), the intermediate conjugate freshly mixed with the unconjugated antibody, and the control immunoconjugate made with an irrelevant anti-AFP immunoglobulin. The difference in tumor size between the treatment groups and the controls was analyzed by Student’s t test (16).

Host Toxicity of the Immunoconjugate. Systemic toxicity of the immunoconjugate was evaluated in BALB/c mice (Harlan Sprague-Dawley, Indianapolis, IN) by following the animals’ body weight change as well as the peripheral white blood counts compared with those receiving the unconjugated drugs. Briefly, a group of 10 animals (5- to 6-wk-old female BALB/c mice) were given a single i.v. injection of the test materials. The animals’ body weights were monitored, and the blood specimens were collected weekly after the injection. The experiments were terminated on Day 21. In monitoring the WBC counts, 40 μl of freshly collected blood were washed with 1 ml of PBS and lysed with 1 ml of lysing buffer (composed of 0.826% ammonium chloride, 0.1% potassium bicarbonate, and 0.0037% EDTA), and the total WBC counts were then determined with a Spectrum III flow cytometer.

RESULTS

Preparation of the Immunoconjugates. Either daunorubicin or doxorubicin can be site specifically conjugated with monoclonal antibody at the carbohydrate moiety via the dextran-bridge linking method (Fig. 1), achieving a level of 20 to 25 drug molecules per antibody. The amino group on the sugar ring of the anthracyclines was first treated with succinic anhydride, and the resulting carboxylic group was then reacted with the amino group of the amino-dextran carrier. Because of the structural difference, elevated reaction temperatures were avoided for doxorubicin. The ratio of substitution falls in the range of 20 to 25 molecules of drug per molecule of dextran. The resulting intermediate conjugate remained water soluble, thus allowing the subsequent antibody conjugation to be conducted in PBS (pH 5.5). The carbohydrate moiety of the antibody can be easily oxidized by treatment with sodium metaperiodate (8, 17). The generation of carbonyl groups was confirmed by reaction with 2,4-dinitrophenyl hydrazine. Antibody aggregation that would normally result from interantibody Schiff base formation does occur. Based on observations with different antibodies, the degree of aggregation varied greatly between antibodies. Such aggregation seems to be related to the pH of the reaction condition, as well as the isoelectric point of the antibodies. In our preparation, this aggregation was most likely generated during the concentrating procedure of the oxidized antibody before the reaction with drug-dextran. It was also noticed that the reaction between the oxidized antibody and the remaining amino groups on the dextran molecule is more efficient than the aggregation formed between antibodies. Based on the HPLC evaluation, the immunoconjugates generally contained greater than 95% of the desired product, and they possessed reasonable stability in the presence of serum. Using labeled drug conjugate, 11.5% of radioactivity was found to be released as small-molecular-weight species after 3 days at 37°C. An additional 10.7% of radioactivity was found in this region after 8 days of incubation. At this time, 8.0% of the radioactivity was also released at medium molecular weight range (presumably was dextran associated); thus, overall 30% of DNR was dissociated from the antibody after 8 days of incubation at 37°C.

Immunoreactivity. Immunoconjugates prepared by this method had a significant retention of antigen-binding activity (Fig. 2). Ag8 antibody was used as a control to determine the background fluorescence in these studies. As indicated, approximately 40 to 50% of the cells stained with NP-4 antibody showed “significant” fluorescence levels over that of Ag8 control. The DOX-dextran-NP-4 immunoconjugate exhibited positive staining of the cells, with equal or slightly reduced intensity with equal or slightly reduced intensity when compared with the parent antibody. This percentage, however, does not imply that the remaining cells were antigen negative (see review in Ref. 18). In contrast, the irrelevant anti-α-fetoprotein antibody and the immunoconjugate constructed with this antibody showed only a minimal binding with this cell line, which apparently was not dose related.

The immunoreactivity of the conjugates was also measured by using the labeled antibody or conjugate on a CEA-affinity column. NP-4 antibody or the DOX-dextran-NP-4 conjugate was radioiodinated with 125I or 131I (NEN, Boston, MA), respectively, by the chloramine-T procedure (15). The immunoconjugates were less reactive with the labeling, and a lower specific radioactivity was generally obtained. However, the labeled materials were found to be 90 to 95% aggregates free by HPLC analysis on a DuPont Zorbax GF-250 column (approx-
Table 1  Tissue distribution of radioiodinated NP-4 and DOX-dextran-NP-4 in GW-39 tumor-bearing nude mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NP-4 (% of ID/g of tissue)</td>
<td>DOX-dextran-NP-4 (% of ID/g of tissue)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gw-39</td>
<td>9.76 ± 3.19*</td>
<td>10.85 ± 3.64</td>
<td>8.36 ± 2.25</td>
<td>12.29 ± 3.43</td>
</tr>
<tr>
<td>Liver</td>
<td>5.45 ± 1.67</td>
<td>5.45 ± 1.74</td>
<td>2.84 ± 0.58</td>
<td>2.85 ± 0.69</td>
</tr>
<tr>
<td>Spleen</td>
<td>4.70 ± 1.87</td>
<td>4.78 ± 1.79</td>
<td>2.24 ± 0.58</td>
<td>2.16 ± 0.51</td>
</tr>
<tr>
<td>Kidney</td>
<td>5.86 ± 1.57</td>
<td>5.64 ± 1.77</td>
<td>2.68 ± 0.30</td>
<td>2.44 ± 0.46</td>
</tr>
<tr>
<td>Lungs</td>
<td>8.27 ± 1.43</td>
<td>8.09 ± 1.19</td>
<td>4.18 ± 1.04</td>
<td>3.90 ± 1.10</td>
</tr>
<tr>
<td>Blood</td>
<td>19.79 ± 4.16</td>
<td>19.84 ± 4.35</td>
<td>9.94 ± 1.58</td>
<td>9.65 ± 1.83</td>
</tr>
<tr>
<td>Bone</td>
<td>2.31 ± 0.39</td>
<td>2.42 ± 0.37</td>
<td>1.43 ± 0.03</td>
<td>1.46 ± 0.09</td>
</tr>
</tbody>
</table>

* Mean ± SD.

Table 2  Tumor/nontumor ratio of NP-4 and DOX-dextran-NP-4 in GW-39 tumor-bearing nude mice on Day 7 after i.v. injection of radioiodinated conjugate or free antibody

<table>
<thead>
<tr>
<th>Tissue</th>
<th>NP-4</th>
<th>DOX-dextran-NP-4</th>
<th>Localization index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>1.34 ± 0.12*</td>
<td>1.96 ± 0.23</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td>Liver</td>
<td>4.79 ± 1.02</td>
<td>6.78 ± 1.44</td>
<td>1.03 ± 0.05</td>
</tr>
<tr>
<td>Spleen</td>
<td>6.09 ± 0.94</td>
<td>8.79 ± 0.61</td>
<td>1.00 ± 0.09</td>
</tr>
<tr>
<td>Kidney</td>
<td>4.98 ± 0.71</td>
<td>7.75 ± 0.79</td>
<td>0.93 ± 0.07</td>
</tr>
<tr>
<td>Lungs</td>
<td>3.25 ± 0.51</td>
<td>4.92 ± 0.62</td>
<td>0.96 ± 0.08</td>
</tr>
<tr>
<td>Bone</td>
<td>9.40 ± 2.13</td>
<td>13.05 ± 3.44</td>
<td>1.06 ± 0.04</td>
</tr>
<tr>
<td>Gw-39</td>
<td>1.46 ± 0.07</td>
<td>1.46 ± 0.07</td>
<td>1.46 ± 0.07</td>
</tr>
</tbody>
</table>

* Tumor/nontumor ratios were calculated by the following formula:

\[
\text{Tumor/nontumor ratio} = \frac{\text{av. % of ID/g in tumor}}{\text{av. % of ID/g in tissue}}
\]

\[
\text{Localization index} = \frac{\% \text{ of ID/g of conjugate in tissue}}{\% \text{ of ID/g of conjugate in blood}}
\]

* Mean ± SD.

Fig. 3. Cytotoxic effects of DOX (○), DOX mixed with NP-4 (♀), DOX-dextran (Δ), DOX-dextran-anti-AFP (□), and DOX-dextran-NP-4 (○) on LoVo cells. The effect of DOX-dextran-NP-4 was also examined on the cells that were preincubated with 50 µg/ml of unconjugated NP-4 (♀).
Table 3  Antitumor effects of DOX, DOX-dextran, and the DOX-dextran + antibody mixture in GW-39 tumor-bearing nude mice

<table>
<thead>
<tr>
<th>DOX injection (µg)</th>
<th>Total dose (mg/kg)</th>
<th>Tumor size (cm³)</th>
<th>% of inhibition</th>
<th>% of change in body wt., final</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial</td>
<td>Final</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.11 ± 0.02</td>
<td>1.35 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>50</td>
<td>0.13 ± 0.03</td>
<td>1.10 ± 0.93</td>
<td>21.8*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.10 ± 0.03</td>
<td>0.23 ± 0.14</td>
<td>-23.7</td>
</tr>
<tr>
<td>DOX-dextran</td>
<td>50</td>
<td>0.08 ± 0.03</td>
<td>1.46 ± 0.64</td>
<td>-9.2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.08 ± 0.05</td>
<td>1.36 ± 0.86</td>
<td>-9.4</td>
</tr>
<tr>
<td>DOX-dextran + NP-4</td>
<td>100</td>
<td>0.07 ± 0.05</td>
<td>1.16 ± 0.73</td>
<td>14.1*</td>
</tr>
</tbody>
</table>

* Tumors were inoculated 8 days before treatment. Conjugates were injected i.p. at 3- to 4-day intervals for a total of 7 injections. The experiment was terminated on Day 24 after the initiation of the treatment. Six animals per group were used.

% Tumor sizes were calculated as h x length x width x depth.

* The percentage of inhibition was calculated as (1 — T/C) x 100, where T is the average absolute tumor increase of the treatment group, and C is the average tumor increase of the controls.

Mean ± SD.

P < 0.5, as compared with saline control.

Table 4  Antitumor effects of DOX-dextran-NP-4 and DOX-dextran-anti-AFP in GW-39 tumor-bearing nude mice

<table>
<thead>
<tr>
<th>DOX-injection (µg)</th>
<th>Total dose (mg/kg)</th>
<th>Tumor size (cm³)</th>
<th>% of inhibition</th>
<th>% of change in body wt., final</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial</td>
<td>Final</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.11 ± 0.02</td>
<td>1.35 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>DOX-dextran-NP-4</td>
<td>Control</td>
<td>1.75</td>
<td>0.10 ± 0.05</td>
<td>0.46 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.04 ± 0.03</td>
<td>1.70 ± 0.74</td>
<td>0.46 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>17.5</td>
<td>0.03 ± 0.20</td>
<td>0.36 ± 0.20</td>
<td>0.36 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>45.0</td>
<td>0.11 ± 0.02</td>
<td>0.49 ± 0.16</td>
<td>81.0*</td>
</tr>
<tr>
<td>DOX-dextran-anti-AFP</td>
<td>Control</td>
<td>0.08 ± 0.03</td>
<td>1.63 ± 0.25</td>
<td>13.5*</td>
</tr>
<tr>
<td></td>
<td>130</td>
<td>0.08 ± 0.00</td>
<td>1.43 ± 0.56</td>
<td>13.5*</td>
</tr>
</tbody>
</table>

* Mean ± SD.

P < 0.001.

P < 0.1.

P < 0.001.

P < 0.5.

Table 5  Toxicological study of the DOX-dextran-NP-4 conjugate in tumor-free BALB/c mice

<table>
<thead>
<tr>
<th>Reagent (mg/kg)</th>
<th>% of body wt. change at termination (vs. control)</th>
<th>% of WBC change at termination</th>
<th>No. of animal deaths</th>
<th>No. of animal deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOX</td>
<td>-14.1 (9)</td>
<td>32.4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>-11.6 (3)</td>
<td>-8.0</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>-1.5 (1)</td>
<td>-40.0</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>DOX-dextran-NP-4</td>
<td>-1.5 (1)</td>
<td>-13.0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parentheses, number.

But insignificant (P < 0.5) inhibition of tumor growth (14.1%).

The specific immunonjugate (DOX-dextran-NP-4) at doses of 5, 50, and 130 µg per injection, equivalent to 1.75, 17.5, and 45 mg/kg, respectively, produced improved antitumor activity as compared with free drug or the intermediate conjugate. As shown in Table 4, the antitumor activity was slightly increased from 70% to 80% when the dose of the immunoconjugate increased from 1.75 to 17.5 mg/kg. A further increase of the immunonjugate to 45 mg/kg did not improve therapeutic efficacy, since an 81% inhibition of tumor growth was observed. These tumor growth inhibitions are significantly different from either the saline control (P < 0.001), the unconjugated drug (P < 0.001), or the control conjugate (P < 0.01). No body weight loss of animals was observed at all dose levels of the specific immunonjugate.

Host Toxicity of the Immunonjugate. Host toxicity of the immunonjugate in comparison to free drug was also evaluated in normal BALB/c mice (Table 5). Unconjugated DOX at a dose of 12 mg/kg caused one animal death of 10 and a 14.1% body weight loss at the termination of the experiment. The toxicity reached its peak approximately 1 wk after the single i.v. introduction of the agent. At this dose level, an 18.4% body weight loss was observed at Day 7 postinjection. However, the animals' body weight gradually recovered. Peripheral WBC toxicity was greatly reduced, so that even a higher dose of the drug (35 mg/kg of the DOX equivalent) could be given to the animals without causing a dramatic body weight reduction (an average loss of 9.4% was observed). However, the tumoricidal activity of the drug was compromised by the dextran conjugation. This is similar to our previous observations for different drug intermediate conjugates (8, 9). Mixing the intermediate conjugate (35-mg/kg DOX dose) with unconjugated antibody (10-fold in weight to the mass of DOX dose) caused a small
levels were not significantly different from the controls under this treatment. A 50% lethality was found in the range of 14 to 16 mg/kg. At the dose of 16 mg/kg, the average body weight loss and the WBC reduction of the surviving animals (n = 3) were 11.6% and 8.0%, respectively, at the termination of the experiment. Therefore, the maximal tolerated dose of DOX is approximately in the range of 10 mg/kg. DOX-dextran-NP-4, similar to FUR-dextran-NP-2 (8), had less host toxicity and caused a minor reduction of the body weight (1.5%) and the WBC count (13%) at the dose of 45 mg/kg (a dose that is already 3-fold higher than the LD<sub>50</sub> of the free DOX).

**DISCUSSION**

Doxorubicin is an important anthracycline antibiotic used in the treatment of a variety of human neoplastic diseases (20, 21). Extensive efforts have been focused on the conjugation of this agent with tumor-reactive antibodies (22, 23), either through the amino sugar moiety or through the C-14 position. The drug could either be attached directly to the antibody (22, 23), through a polymeric bridge such as dextran (24, 25) or PGA (26, 27), or through a short polypeptide (28). Linkages such as amide bonds have been used widely (22); others such as acid-labile cis-aconitic amide (29) and hydrazone derivatives (30) also have been described recently. In this study, we undertook the site-specific conjugation of DOX with antibody via the amino-dextran bridge procedure that we described previously, where both methotrexate and fluorouridine were conjugated with murine monoclonal antibody at a substitution level in the range of 20 to 40 drug molecules per antibody (7, 8). In this study, DOX was first conjugated with the dextran carrier through an amide-type of linkage, and the entire drug-intermediate conjugate was then linked to the carbohydrate moiety of the antibody through an alkylamine linkage that formed between the amino group of the amino-dextran carrier and the carbonyl group of the oxidized carbohydrate. The amide linkage between the drug and the dextran provides a plausible site for proteolytic cleavage to release the free drug necessary for pharmacological activity. Gallego et al. (22) indicated that anthracyclines linked to an antibody via succinic anhydride chemistry have resulted in an immunoconjugate essentially inactive toward the target cells in an in vitro assay. This finding further supports the conclusions of Deprez-De Campeneere et al. (28) that the efficiency of lysosomal hydrolysis to release intact DNR is greatly dependent on the length of the spacer arm between the drug and the protein. In their study, no active DNR can be detected after lysosomal digestion of the conjugate prepared by a direct linkage between protein and succinylated DNR. The release of intact DNR was increased proportionally to the distance between drug and protein; thus, a spacer arm was required to allow the enzymatic hydrolysis to take place. By choosing a bridging system in our study, a spacer arm is present in the expectation that a better chance of proteolytic cleavage could take place.

Using this procedure, approximately 20 to 25 molecules of drug could be attached to each immunoglobulin without significant reduction of the antigen-binding activity of the antibody. Such an immunoconjugate also demonstrated retention of targeting to the GW-39 tumor xenograft. Although the immunoconjugates expressed less toxicity in the in vitro assay with an antigen-positive cell line, the in vivo antitumor activity of the conjugate, on the contrary, was much superior to that of the free drug. Free DOX at a dose of 17.5 mg/kg produced a marginal 21.8% inhibition of the GW-39 tumor growth, while the body weight of the animals was reduced by 23.7%. In contrast, the specific immunoconjugate (DOX-dextran-NP-4), at the equal drug dose, caused a higher antitumor effect (80% versus 21.8%) and no systemic toxicity. Furthermore, the dose of the conjugate could be increased further to 45 mg/kg without any evidence of host toxicity. However, no significant increase in antitumor activity was demonstrated in this model. This observation may be due to the saturation of the tumor or due to the limited penetration of the antibody in the tumor, leaving a fraction of the tumor cells difficult to be bound by the conjugate.

It is an obvious advantage to have an immunoconjugate that carries a high number of drug molecules per antibody molecule. In order to achieve this high substitution level of drug-antibody conjugate without significantly impairing its antigen-binding activity, a polymer molecule of appropriate size has been used as the intermediary in indirect conjugation methods. As discussed already, polymers such as oxidized dextran, PGA, human serum albumin, and carboxymethyl-dextran have been utilized in recent years (24, 26, 31). It was observed, in the studies of Garnett et al. (31) and Endo et al. (32, 33), that conjugates with increased levels of drug substitution through the use of an intermediate carrier were superior to the use of direct conjugates.

Host toxicity of the conjugate was also evaluated in tumor-free BALB/c mice and compared with that of free drug. The LD<sub>50</sub> dose of DOX was approximately equal to 12 mg/kg of body weight, and the LD<sub>50</sub> dose was within the range of 14 to 16 mg/kg. At the endpoint of the study (Day 21), animals that received toxic doses of free DOX (12, 16, and 20 mg/kg) still showed a body weight loss in the range of 10 to 15% when compared with the controls. The corresponding percentage of change of WBC in these animals was dose related. The WBC counts of animals that survived the high dose of DOX (20 mg/kg) recovered but were still significantly lower than the pretreatment WBC counts. When the animals were given DOX-dextran-NP-4 at a dose at least 3-fold greater than the LD<sub>50</sub> of free DOX, an average of only a 1.5% body weight loss and an insignificant 13% reduction of WBC count were observed. These findings are similar to those of FUR-dextran-NP-2 (8) and indicated that the immunoconjugate is less toxic than the free drug; a dose of at least 3 to 4 times that of free drug could be tolerated well.

Similar to our previous observation on methotrexate- and FUR-dextran (8, 9), the intermediate conjugates, as well as the intermediate conjugate mixed with free antibody, failed to produce the same antitumor effects at the same dose level of the specific immunoconjugate, demonstrating the importance of the covalent linkage between DOX-dextran and antibody.

In summary, our results show that amino-dextran can be used as an intermediate drug carrier for antibody conjugation in order to increase the drug substitution level. The resulting immunoconjugate exerted higher antitumor effects as compared with those of free drug and the drug-dextran intermediate at an equal dose level. Since the intermediate conjugate did not cause an equal extent of tumor growth inhibition, the higher antitumor effects of the specific immunoconjugate evidently are not due to free DOX that may be released due to extracellular degradation. Furthermore, the insignificant marginal inhibition observed for DOX-dextran-anti-AFP at a dose of 45 mg/kg further supports the view that the antitumor effects of the
specific immunoconjugate are indeed due to specific antibody delivery.

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Anthracycline Immunoconjugates Prepared by a Site-specific Linkage via an Amino-Dextran Intermediate Carrier

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