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

Lisa B. Shih, David M. Goldenberg, Hong Xuan, Helen Lu, Robert M. Sharkey, and Thomas C. Hall

Garden State Cancer Center and Center for Molecular Medicine and Immunology, Newark, New Jersey 07103

ABSTRACT

Anthracycline, either daunomycin or doxorubicin, was site specifically attached to the carbohydrate moiety of a monoclonal anticalcinoembryonic antigen antibody by using amino-dextran as the intermediate carrier. The reaction resulted in an immunocjugate 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 immunocjugate was cytotoxic to the target cells, as examined by the 35Selenomethionine incorporation studies, and remained efficient for targeting a human colonie tumor (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 it 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 immunocjugate 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 immunocjugates with a high substitution level as well as preferred immunological properties. The chemical modifications, tumor targeting, and inhibitory effects of these immunocjugates 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 jg/ml), streptomycin (100 jg/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 anticalcineembryonic 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 immunocjugate.

GW-39, a human colon carcinoa, 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 jmol), was dissolved in 0.6 ml of anhydrous dimethylformamide. To this solution, 7.5 jmol of triethylamine were added, followed by 1.9 mg of succinic anhydride. The mixture was reacted in the dark 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

Received 1/31/91; accepted 5/31/91.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported in part by USPHS Grant CA 39841 from the NIH and by Grant 89-240360-6 from the New Jersey Commission on Science and Technology.

2 To whom requests for reprints should be addressed, at the Center for Molecular Medicine and Immunology, 1 Bruce St., Newark, NJ 07103.
SITE-SPECIFIC CONJUGATION OF ANTHRACYCLINES

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

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; M, 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 conjugate (drug-dextran). An Apex octadecyl reverse-phase column (Jones Chromatography, Littleton, CO) was used to evaluate the amount of unconjugated DOX present in the sample. Using 1% triethylamine acetate (pH 4) and acetonitrile (70:30) as mobile phase, DOX-dextran-NP-4 and unconjugated DOX were eluted at 6.0 and 6.7 min, respectively. A very minimal amount of DOX (2 to ~4%) was found to be present in the conjugate as evaluated under the detection at 482 nm.

In Vitro Stability of the Conjugate. [3H]DNR (NEN, Boston, MA) was used to prepare the radioactive conjugate. The conjugate was sterilized by passing through a syringe filtration disc and mixed with 9 parts of sterile mouse serum. The mixture was then incubated at 37°C 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.

Immunoreactivity of the Conjugates. The immunoreactivity of the conjugates was examined by flow cytometry of the target cells. Briefly, 1 x 10^6 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 125I 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 fractionation column equipped with an in-line radioactivity detector (Beckman, Fullerton, CA) to determine radio purity. The labeled conjugate (25 µCi) was then coinjected with 10 ^6Ci 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 75 selenomethionine 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 x 10^6 cells/ml. One hundred µl of the cell suspension were added...
6-wk-old female BALB/c mice) were given a single i.v. injection of the conjugate upon storage), as well as the peripheral white blood counts compared with those of freshly collected blood were washed with 1 ml of PBS and lysed 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 75Selenomethionine incorporation,

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

Cycloheximide at 50 μM was used as the positive-keeping 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 cm³. The calculated tumor volumes closely correlated with the actual tumor weight. Host toxicity was followed by body weight loss. The animals' body weights were monitored, and the remaining amino groups on the dextran molecule is 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 immunonoconjugates 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 compared with the parent antibody. The DOX-dextran-NP-4 immunoconjugate also 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.

RESULTS

Preparation of the Immunoconjugates. Either daunorubicin or doxorubicin can be site specifically conjugated with monoclonal antibody at the carbohydrate moieties 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 immunonoconjugates 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.
Table 1  Tissue distribution of radiiodinated 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>NP-4 (% of ID/g of tissue)</td>
<td>DOX-dextran-NP-4 (% of ID/g of tissue)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gw-39</td>
<td>9.76 ± 3.19</td>
<td>8.36 ± 2.23</td>
<td>10.85 ± 3.64</td>
<td>12.29 ± 3.43</td>
</tr>
<tr>
<td>Liver</td>
<td>5.45 ± 1.67</td>
<td>2.84 ± 0.58</td>
<td>5.45 ± 1.74</td>
<td>2.85 ± 0.69</td>
</tr>
<tr>
<td>Spleen</td>
<td>4.70 ± 1.87</td>
<td>2.24 ± 0.58</td>
<td>4.78 ± 1.79</td>
<td>2.16 ± 0.51</td>
</tr>
<tr>
<td>Kidney</td>
<td>5.86 ± 1.57</td>
<td>2.68 ± 0.30</td>
<td>5.64 ± 1.77</td>
<td>2.44 ± 0.46</td>
</tr>
<tr>
<td>Lungs</td>
<td>8.27 ± 1.43</td>
<td>4.18 ± 1.04</td>
<td>8.09 ± 1.19</td>
<td>3.90 ± 1.10</td>
</tr>
<tr>
<td>Blood</td>
<td>19.79 ± 4.16</td>
<td>9.94 ± 1.58</td>
<td>19.84 ± 4.35</td>
<td>9.65 ± 1.83</td>
</tr>
<tr>
<td>Bone</td>
<td>0.30 ± 0.24</td>
<td>0.68 ± 0.09</td>
<td>2.42 ± 0.37</td>
<td>1.46 ± 0.09</td>
</tr>
</tbody>
</table>

* Localization index

* 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 radioidinated conjugate or free antibody.

Values are the average of 4 animals.

| Tissue   | NP-4 | DOX-dextran-NP-4 | Localization index
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>1.34</td>
<td>1.96</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td>Liver</td>
<td>4.79</td>
<td>6.78</td>
<td>1.03 ± 0.05</td>
</tr>
<tr>
<td>Spleen</td>
<td>6.09</td>
<td>8.79</td>
<td>1.00 ± 0.06</td>
</tr>
<tr>
<td>Kidney</td>
<td>4.98</td>
<td>7.75</td>
<td>0.93 ± 0.07</td>
</tr>
<tr>
<td>Lungs</td>
<td>3.25</td>
<td>4.92</td>
<td>0.96 ± 0.08</td>
</tr>
<tr>
<td>Bone</td>
<td>9.40</td>
<td>13.05</td>
<td>1.06 ± 0.04</td>
</tr>
<tr>
<td>Gw-39</td>
<td>1.46</td>
<td>1.46 ± 0.07</td>
<td></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 (△), 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 (●).

Immediately 100,000 cpm of the radioactivity were evaluated. The immunoconjugates retained the antibody activity, in that 68.7% of the radioactivity bound to the CEA-affinity column, as compared with 71.3% for labeled NP-4 antibody.

Tumor Localization of the Immunoconjugates. The tissue distribution of the labeled NP-4 and DOX-dextran-NP-4, expressed as the percentage of injected dose per gram of tissues, is given in Table 1. The similarity between the antibody and the conjugate, in terms of tumor accretion (from Day 1 to Day 3), and the slower elimination of these two materials from this tissue after Day 3, compared with that of normal tissues, indicate the effectiveness of the tumor targeting of both materials. The tumor/nontumor ratios, as well as the localization index of these two reagents on Day 7, are provided in Table 2, which indicate the similar tumor-targeting ability of the conjugate and the unconjugated antibody.

In Vitro Cytotoxicity. The *in vitro* cytotoxicity of the DOX-dextran-NP-4, as evaluated on LoVo cells by following protein synthesis, is shown in Fig. 3. The IC50 was approximately 0.25 μg/ml for unconjugated DOX. Mixing DOX and NP-4 did not alter the cytotoxic activity of the drug. In microscopic evaluation, the orange fluorescence of DOX accumulated efficiently into the nuclear region as early as 30 min after coincubation of the DOX and antibody with LoVo cells, while the antibody (visualized by the fluorescein isothiocyanate-labeled second antibody) was predominately located on the surface of the cells (data not shown), indicating that these two agents acted independently in this case. This observation indicated that a non-specific complexion between these two agents, as has been reported in another antibody system (19), may not exist in this case. The IC50 for the immunoconjugates was higher than for unconjugated DOX (approximately in the range of 2 to 30 μg/ml). Based on the observation that the cytotoxicity of the conjugate was noticeably blocked by preincubation of the cells with 50 μg/ml of the unconjugated antibody, it can be concluded that the cytotoxic activity demonstrated by the conjugate was mainly mediated through an antibody-antigen interaction instead of the free DOX that may have been present in the sample.

Tumoridal Effects of the Conjugates. The antitumor efficacy of unconjugated drug and DOX-dextran-NP-4 was evaluated on established GW-39 human colon tumor xenografts (Tables 3 and 4). Therapy was started 8 to 9 days after transplantation, when the average size of the tumors was approximately 0.1 g. A protocol of multiple i.p. injections of the testing materials was selected, in the expectation that a higher blood level of the conjugates could be maintained, thus providing a higher availability of the conjugates to the tumor. Increasing dose levels of the free drug, intermediate conjugate (DOX-dextran), and the specific conjugate were examined. In these studies, a saline control was included in each experiment to serve as the standard for interexperiment comparisons. Those studies with control tumors growing to a similar average size were compared in Table 3, while those with control tumors growing more variably were individually compared in Table 4. As indicated in Table 3, free DOX at a dose of 50 μg per injection (*i.e.*, 17.5 mg/kg) caused a 21.8% inhibition of tumor growth. Under this treatment, an average of 23.7% of body weight loss was observed, 3, free DOX at a dose of 50 μg per injection (i.e., 17.5 mg/kg) caused a 21.8% inhibition of tumor growth. Under this treatment, an average of 23.7% of body weight loss was observed.
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., %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial</td>
<td>Final</td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td></td>
<td>0.11 ± 0.02⁴</td>
<td>1.35 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>DOX</td>
<td>50</td>
<td>17.5</td>
<td>0.13 ± 0.03</td>
<td>1.10 ± 0.93</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>35.0</td>
<td>0.10 ± 0.03</td>
<td>0.23 ± 0.14</td>
</tr>
<tr>
<td>DOX-dextran</td>
<td>50</td>
<td>17.5</td>
<td>0.08 ± 0.03</td>
<td>1.46 ± 0.64</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>35.0</td>
<td>0.08 ± 0.05</td>
<td>1.36 ± 0.86</td>
</tr>
<tr>
<td>DOX-dextran + NP-4</td>
<td>100</td>
<td>35.0</td>
<td>0.07 ± 0.05</td>
<td>1.16 ± 0.73</td>
</tr>
</tbody>
</table>

⁴ 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.

⁵ 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.

Day 17 (n = 3).

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., %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial</td>
<td>Final</td>
<td></td>
</tr>
<tr>
<td>DOX-dextran-NP-4</td>
<td></td>
<td>0.11 ± 0.02⁷</td>
<td>1.35 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>Control 5</td>
<td>1.75</td>
<td>0.10 ± 0.05</td>
<td>0.46 ± 0.25</td>
<td>70.9⁸</td>
</tr>
<tr>
<td>Control 50</td>
<td>17.5</td>
<td>0.04 ± 0.03</td>
<td>1.70 ± 0.74</td>
<td>80.6⁹</td>
</tr>
<tr>
<td>Control 130</td>
<td>45.0</td>
<td>0.13 ± 0.02</td>
<td>2.13 ± 0.72</td>
<td>81.0⁹</td>
</tr>
<tr>
<td>DOX-dextran-anti-AFP</td>
<td>0.08 ± 0.03</td>
<td>1.63 ± 0.25</td>
<td>13.5⁹</td>
<td>1.6</td>
</tr>
<tr>
<td>Control 130</td>
<td>45.0</td>
<td>0.08 ± 0.01</td>
<td>1.43 ± 0.56</td>
<td></td>
</tr>
</tbody>
</table>

⁶ All P values were calculated by comparing the tumor size to the respective saline control.

Mean ± SD.

P < 0.001.

P < 0.01.

P < 0.01.

P < 0.5.

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

Ten animals per group were used. The testing material was given via a single i.v. injection, and toxicity was followed to Day 21.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>% of body wt. change at termination (vs. control)</th>
<th>% of WBC change at termination</th>
<th>No. of animal deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOX</td>
<td>-14.1 (9)⁴</td>
<td>32.4</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>-11.6 (3)</td>
<td>-8.0</td>
<td>7</td>
</tr>
<tr>
<td>20</td>
<td>-1.5 (1)</td>
<td>-40.0</td>
<td>9</td>
</tr>
<tr>
<td>DOX-dextran-NP-4</td>
<td>-1.5 (10)</td>
<td>-13.0</td>
<td>0</td>
</tr>
</tbody>
</table>

⁴ Numbers in parentheses, number.

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 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 immunoconjugate 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 Immunoconjugate. Host toxicity of the immunoconjugate 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...
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 hydrazine 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 fluorouracil 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 succinyl anhydride chemistry have resulted in an immunconjugate essentially inactive toward the target cells in an <i>in vitro</i> assay. This finding further supported 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 immunomonomer conjugate also demonstrated retention of targeting to the GW-39 tumor xenografts. Although the immunomonomer conjugates expressed less toxicity in the <i>in vitro</i> assay with an antigen-positive cell line, the <i>in vivo</i> 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 immunomonomer conjugate 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 carboxymethylidextran 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 immunomonomer 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 immunomonomer, 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 immunomonomer 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 immunomonomer 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.

ACKNOWLEDGMENTS

We are grateful to D. Pawlyk and Maggie Garcia for their assistance.

REFERENCES


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

Lisa B. Shih, David M. Goldenberg, Hong Xuan, et al.


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/51/16/4192

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/51/16/4192. Click on “Request Permissions” which will take you to the Copyright Clearance Center's (CCC) Rightslink site.