Antitumor Activity of Carcinoma-reactive BR96-Doxorubicin Conjugate against Human Carcinomas in Athymic Mice and Rats and Syngeneic Rat Carcinomas in Immunocompetent Rats

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ABSTRACT

The internalizing monoclonal antibody BR96 was conjugated to the anticancer drug doxorubicin (DOX) using an acid-labile hydrazone bond to DOX and a thioether bond to the monoclonal antibody. The resulting conjugate, termed BR96-DOX, binds to a tumor-associated Lewis 7 antigen that is abundantly expressed on the surface of human carcinoma cells. BR96-DOX binds to RCA, a human colon carcinoma cell line, and BN7005, a transplantable colon carcinoma induced in a Brown Norway (BN) rat by 1,2-dimethyl-hydrazine. BR96-DOX produces cures of established s.c. RCA human colon carcinoma in athymic mice and rats. BR96-DOX also cured both s.c. and intrahepatic BN7005 tumors in immunocompetent BN rats. Unconjugated DOX, given at its maximum tolerated dose, and matching doses of nonbinding IgG-DOX conjugate were not active against RCA or BN7005 carcinomas. An anticonjugate antibody response was produced in BN rats treated with BR96-DOX. However, this could be largely prevented by administering the immunosuppressive drug deoxyspergualin. These results confirm the concept of antibody-directed therapy in models in which the targeted antigen is expressed both in normal tissues and tumors. The findings in BN7005 further demonstrate efficacy of BR96-DOX therapy in a model in which the tumor is syngeneic and the host is immunocompetent.

INTRODUCTION

MAbs 3 to tumor-associated antigens have been used with variable success in the treatment of cancer. With the exception of treatment of minimal residual disease colon carcinoma, unmodified MAbs have shown little antitumor activity. MAbs have also been used to deliver a variety of toxic moieties to malignant cells (2-4). Antigen-specific activity has been demonstrated in vitro and in nude mouse models with several such conjugates. However, to date, the clinical efficacy of MAb-mediated delivery has been demonstrated definitively only for radiolabeled MAb conjugates used in the therapy of B cell lymphoma (5,6).

Several factors contribute to the lack of clinical predictability of the models commonly used to evaluate MAb conjugates. Typically, treatment starts shortly after xenografting of human tumors to congenitally athymic (nude) mice when the tumor burden is very small, and conclusions about efficacy are commonly based on a delay in tumor outgrowth rather than on regression or cure of established tumors. The tumors used are usually sensitive to the free drug administered by an optimized route and schedule, although activity of the drug may not be observed at "conjugate equivalent" doses. The majority of MAbs identified to date and, therefore, conjugates of these MAbs are tumor selective rather than tumor specific in patients because binding to cells of some normal tissues is typically observed. However, the preclinical models typically used do not address the issue of tumor-selective targeting because rodent do not, for the most part, express the target antigen in normal tissues. Furthermore, patients in Phase I/II clinical trials usually have bulky tumors, into which immunoconjugates penetrate poorly (7), and the tumors are frequently resistant to chemotherapeutic drugs as a consequence of extensive prior therapy. In addition, whereas an antibody response to the conjugate is common in patients, it cannot occur in athymic rodents.

MAb BR96 recognizes a Le 7-related epitope that is expressed (>100,000 molecules/cell) at the surface of cells from the majority of human cancers of breast, colon, and lung (8,9). The conjugate, termed BR96-DOX, was prepared using a chimeric (mouse-human) form of murine BR96 (10) and the DOX derivative 6-maleimidocaproyl DOX hydrazone so that it contained both an acid labile bond and a thioether linker (11,12). Upon binding to the tumor cell surface, BR96-DOX internalizes into the acidic environment of lysosomes/endosomes and liberates DOX. The BR96-DOX conjugate was shown to produce cures of antigen-expressing tumors, to be at least 8-fold as potent as the unconjugated parent drug DOX, and to be active at a dose equivalent to 5% of its MTD in athymic mice (11).

The antigen to which BR96 binds is not expressed in mice. It is, however, detectable in the gastrointestinal epithelium of humans, dogs, and rats. It was encouraging, therefore, that cures were obtained also in nude rats xenotransplanted with DOX-sensitive human lung tumors (11). Although athymic rats provide an appropriate model for conjugate efficacy and toxicity, they lack the ability to produce an anticonjugate response. Athymic animals may also mount a limited host response to xenotransplanted tumors, which may facilitate regression after an initial damage inflicted by the immunoconjugate (13). Therefore, a model of a syngeneic tumor in immunocompetent animals that expresses the target antigen in normal tissues would reflect the clinical situation more closely.

This study was designed to address several of these issues. The efficacy of BR96-DOX was investigated against several types of colon tumor models. These were s.c. implanted RCA human colon xenografts studied in athymic mice and rats and a rat colon carcinoma, BN7005, which was evaluated as a s.c. metastasis model and an experimental liver metastasis model in syngeneic, immunocompetent rats. In the rat models, the BR96 MAb was tumor selective rather than tumor specific. The effect of an anticonjugate response on the efficacy of BR96-DOX against BN7005 carcinomas was also evaluated.

MATERIALS AND METHODS

MAbs and Immunoconjugates

MAb BR96 has been described previously (8,9). Human IgG (Rockland Inc., Gilbertsville, PA) was used to produce nonbinding control immunoconjugates. Immunoconjugates consisting of BR96 (BR96-DOX) or human IgG (IgG-DOX) conjugated to DOX were prepared as described previously (12).
Assay for Rat Antibodies against BR96-DOX

To evaluate rat anticonjugate antibody responses, sera of blood samples drawn from treated rats were assayed in an ELISA measuring specific binding of rat serum antibodies to chimeric BR96-DOX conjugate expressed as $A_{450nm}$ at a dilution of 1:100.

Carcinoma Lines

RCA is a human adenocarcinoma of the colon obtained from M. Brattain (Medical College of Ohio, Toledo, OH). BN7005 is a rat adenocarcinoma of the colon, induced by 1,2-dimethylhydrazine in a BN rat and cloned by limiting dilution in the absence of selection pressure.

In Vitro Cytotoxicity Assays

Antigen-specific cytotoxicity was evaluated as described previously (14). Briefly, monolayer cultures of colon carcinoma cells were harvested using trypsin-EDTA (Life Technologies, Inc., Grand Island, NY), and the cells were resuspended to $1 \times 10^6$/ml RPMI 1640 containing 10% heat-inactivated FCS. The cells were added to flat-bottomed 96-well microtiter plates (0.1 ml/well) and incubated overnight at 37°C in a humidified atmosphere of 5% CO$_2$ in air. Media were removed from the plates, and serial dilutions of BR96-DOX, IgG-DOX, or DOX were added to each of the wells. Quadruplicate samples were assayed. The cells were exposed to the drug or conjugates for 2 h at 37°C in a humidified atmosphere of 5% CO$_2$ in air. The drug or conjugate was then removed, and the cells were washed three times with RPMI and cultured in RPMI containing 10% heat-inactivated FCS. Approximately 48 h later, the cells were pulsed for 2 h with 1.0 µCi/well of $[^3H]$thymidine (New England Nuclear, Boston MA). The media were removed, and trypsin was added to the wells. The cells were harvested (Skatron Instruments, Sterling, VA) onto glass fiber filter mats and dried, and filter-bound $[^3H]$thymidine radioactivity was determined (β-scintillation counter, Pharmacia LKB Biotechnology, Piscataway, NJ). Inhibition of $[^3H]$thymidine uptake was determined by comparing the mean cpm for treated samples with the mean cpm of the untreated control.

Experimental Animals

Immunocompetent inbred male rats, 3–5 months old, of the BN strain were obtained from a closed colony maintained at the Wallenberg Laboratory. Athymic female mice of BALB/c background and athymic (Rowett) female rats were obtained from Harlan Sprague Dawley. Animals received food and water ad libitum.

Tumor Models

s.c. tumors were measured in two perpendicular directions at weekly or biweekly intervals using calipers. Tumor size was defined as follows: $l \times w^2/2$, where $l =$ measurement of longest axis (mm) and $w =$ measurement of axis perpendicular to $l$ (mm). Data are presented as median tumor size. Antitumor activity is expressed in terms of TVDDs as described previously (15). A tumor growth delay equivalent to ≥3.3 TVDD was considered evidence of biological activity. PR reflects a decrease in tumor volume to ≤50% of the initial tumor volume; CR refers to a tumor that has regressed completely and is not palpable for a period of time equal to the tumor volume doubling time; and cure is defined as an established tumor that has regressed completely and that, after regression, is not palpable for a period of time ≥10 TVDTs.

Human Carcinoma Line RCA

Studies in athymic mice used s.c. tumors maintained by in vivo passage as described previously (15). For athymic rat studies, RCA cells were harvested in logarithmic growth using trypsin-EDTA and washed in serum-free medium, and $5 \times 10^6$ cells were injected s.c. in the left axillary region.

Rat Carcinoma Line BN7005

s.c. BN7005 Tumors. Tumors were excised and minced in PBS to obtain a single cell suspension, which was inoculated s.c. in the thigh of the right hind leg.

Intrahepatic BN7005 Tumors. Human colon carcinomas commonly metastasize to the liver, whereas those of rats do not. To evaluate whether a therapeutic response could be achieved against BN7005 growing in the liver, a model was developed in which approximately $10^6$ BN7005 cells were implanted under the capsule of two liver lobes. In untreated animals, tumor nodules were visible at laparotomy 7 days after grafting and enlarged rapidly, spreading into the peritoneal cavity and mesenteric lymph nodes yielding ascites and killing recipients within 3–4 weeks. Tumor growth was determined at laparotomy by liver inspections and measurements of perpendicular diameters of intrahepatic tumors, which were detected by a distinct, light color contrasting to the normal liver tissue.

Therapy

Tumors were staged to various sizes prior to therapy. Athymic mice and rats received all therapy i.v., whereas BN rats received DOX i.v. and BR96-DOX and IgG-DOX i.p. Treatments were performed on an individual body weight basis; doses were presented as both mg/m² of MAb and equivalent DOX (16). Conjugates typically contained 1 mg of DOX per 35 mg of MAb. To clarify whether the anticipated generation of an anticonjugate response toward BR96-DOX might reduce the efficacy of BR96-DOX therapy, experiments were performed in which BN rats were treated concurrently with DSG, a drug shown previously to suppress the appearance of an antibody response to analogous conjugates in mice (17, 18). DSG was administered i.p. in 11 daily doses of 30 mg/m² beginning one day after the first day of BR96-DOX therapy. In one experiment, a second round of BR96-DOX therapy, with or without DSG, was given when tumors had regrown following BR96-DOX-induced PR.

RESULTS

Antigen-specific Cytotoxicity of BR96-DOX Conjugates in Vitro. The in vitro potency and specificity of BR96-DOX was evaluated against RCA and BN7005 carcinoma cells following a 2-h drug or conjugate exposure. As shown in Table 1, BR96-DOX demonstrated antigen-specific cytotoxicity against both the RCA and BN7005 colon carcinoma lines. The BR96-DOX conjugate was more potent than a nonbinding IgG-DOX conjugate prepared using the same linker chemistry. The BR96-DOX was approximately 10-fold less potent than unconjugated DOX following a 2-h exposure in vitro.

Activity of BR96-DOX against s.c. RCA Human Colon Tumors in Athymic Mice and Rats. Previous studies have reported that RCA colon tumors in athymic mice were cured by BR96-DOX, although the xenografts were insensitive to unconjugated DOX (11). Antitumor activity of BR96-DOX was shown to be antigen specific because a nonbinding IgG-DOX conjugate was not active. In the study presented here, the activity of BR96-DOX against established s.c. RCA colon tumors was evaluated in both athymic mice and rats. In each case, unconjugated DOX was administered at its MTD; 24 and 12 mg/m² in mice and rats, respectively. BR96-DOX was administered to both mice and rats at a dose of 420 mg/m² BR96-DOX (12 mg/m² equivalent DOX). In athymic mice (Fig. 1), the BR96-DOX conjugate produced cures of established RCA xenografts, whereas unconjugated...
DOX administered at twice that dose was not active (<3.3 TVDD). In the RCA colon tumor model in athymic mice, BR96-DOX was more active and more potent than unconjugated DOX. The athymic mouse model, although an appropriate model for demonstrating distal site antigen-specific activity, does not address the issue of normal tissue expression of the targeted antigen. Previous studies demonstrated that BR96-DOX produced cures of established DOX-sensitive human lung tumor xenografts in athymic rats (11). Rats, like humans and in contrast to mice, have been shown by immunohistochemistry to bind BR96; the normal tissue reactivity occurs primarily on cells of the gastrointestinal tract; esophagus, stomach, intestine, and pancreas (acinar cells; Refs. 8 and 11). The athymic rat, therefore, provides a unique model to evaluate the antitumor activity of BR96-DOX against DOX-insensitive human colon tumors in a system in which the targeting MAb is tumor selective rather than tumor specific. As shown in Fig. 1B, the BR96-DOX conjugate, administered at a dose equivalent to the MTD of unconjugated DOX, demonstrated a significant tumor growth delay, with 50% CRs and 50% PRs. In contrast, unconjugated DOX administered at the same dose was not active. Therefore, BR96-DOX was active and tolerated in a model of a human DOX insensitive

Table 2 Antigen-specific antitumor activity of BR96-DOX against s.c. BN7005 syngeneic rat colon carcinoma

<table>
<thead>
<tr>
<th>Dose/injection (mg/m²)</th>
<th>Treatment</th>
<th>MAb</th>
<th>DOX</th>
<th>Complete</th>
<th>Partial</th>
<th>Total responding/ no. of Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>BR96-DOX</td>
<td>2.5</td>
<td>0</td>
<td>0</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>175</td>
<td>5.0</td>
<td>1</td>
<td>0</td>
<td>1/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>350</td>
<td>10.0</td>
<td>3</td>
<td>0</td>
<td>3/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>420</td>
<td>12.0</td>
<td>3</td>
<td>0</td>
<td>9/13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>420</td>
<td>IgG-DOX</td>
<td>12.0</td>
<td>0</td>
<td>0</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>420</td>
<td>DOX</td>
<td>12.0</td>
<td>0</td>
<td>0</td>
<td>0/8</td>
<td></td>
</tr>
<tr>
<td>18.0b</td>
<td>24.0b</td>
<td>0</td>
<td>0</td>
<td>0/20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Treatments administered every 4 days for 3 injections.

b Toxicity observed as weight loss >15% and hind leg paralysis.

Table 3 Response of established intrahepatic isografts of the BN7005 colon carcinoma to BR96-DOX administered i.p. on four occasions

<table>
<thead>
<tr>
<th>Wk after tumor isografting</th>
<th>Treatmenta</th>
<th>Proportion with tumor</th>
<th>Tumor diameter (mm)</th>
<th>Proportion with tumor</th>
<th>Tumor diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>6/6</td>
<td>6/6</td>
<td>≈ 3 ± 1.4</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td>BR96-DOX (12 mg/m² DOX)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proportion with tumor</td>
<td>6/6</td>
<td>6/6</td>
<td>≈ 4 ± 3</td>
<td>0/6</td>
<td>ND</td>
</tr>
<tr>
<td>Tumor diameter (mm)</td>
<td>0/6</td>
<td>0/6</td>
<td>ND</td>
<td>0/6</td>
<td>ND</td>
</tr>
</tbody>
</table>

a First day of therapy was considered day 1; treatments were administered on days 1, 4, 7, and 11.
b Sacrificed on day 21 because all rats in this group had large tumors in the liver, which spread to the parietoneal wall and mesenteric lymph nodes.
c For each rat, the mean diameter of each tumor of two liver tumors was calculated and averaged. Mean ± SE for each group is shown.
d ND, not done.
e One rat died with multiple lung metastases but no liver tumors. The remaining five rats were sacrificed 5 weeks after the start of therapy and determined to be tumor free on necropsy.
Antitumor Activity of BR96-DOX against s.c. Rat Colon Carcinoma BN7005. Although athymic rats provide an appropriate model for conjugate efficacy and toxicity, they may also mount a limited host response to xenotransplanted tumors, which may facilitate regression after an initial damage inflicted by the immunoconjugate (13). In addition, athymic rats lack the ability to produce an anticonjugate response and cannot be used to address issues of altered conjugate clearance, pharmacokinetics, and tumor localization when anticonjugate antibodies are present. Therefore, a model of a syngeneic tumor in immunocompetent animals expressing the target antigen in normal tissues would reflect the clinical situation more closely. The BN7005 rat colon carcinoma, derived originally from BN rats, was used to address these issues. Treatment of established BN7005 s.c. tumors with BR96-DOX at a dose of 420 mg/m² (12 mg/m² equivalent DOX) administered every 4 days for a total of three injections resulted in antitumor activity equivalent to ≳6.7 TVDD; 3 of 5 rats were cured of their tumors (Fig. 2). As shown, BN7005 tumors were not sensitive to unconjugated DOX because treatment with the MTD of 12 mg/m² did not result in tumor regressions or even a tumor growth delay. The activity of BR96-DOX against established BN7005 tumors was antigen specific; a matching dose of 420

Fig. 3. Macroscopic appearance of rat BN7005 colon carcinoma growing in two liver lobes. A, livers prior to therapy; B, livers 21 days after treatment with BR96-DOX; C and D, liver from untreated control rat on days 7 and day 21, respectively. T, tumor; S, scar tissue.
mg/m² of nonbinding IgG-DOX conjugate (12 mg/m² equivalent DOX) was not active.

The antitumor response of BN7005 to BR96-DOX was dose-dependent (Table 2). At doses of BR96-DOX of 90 and 175 mg/m², administered every 4 days for a total of three injections, 0 of 5 and 1 of 5 tumors regressions were observed, respectively. At 350–420 mg/m², administered every 4 days for a total of three injections, 60–70% tumor regressions were observed (3 of 5 and 9 of 13). A therapeutic effect was not seen with control IgG-DOX conjugates administered at the same dose and schedule. BN7005 tumors were not sensitive to unconjugated DOX, even when it was administered at doses above the MTD. Previous experiments (11) have demonstrated that unconjugated BR96 MAb was not active against established tumors and that mixtures of MAb BR96 and DOX (at the MTD) were no more active than DOX administered alone.

Regressions and Cures of Intrahepatic BN7005 Colon Carcinomas following Treatment with BR96-DOX. BN7005 cells were injected into two liver lobes, and treatment was initiated 7 days later (four treatments given 3 days apart). Although untreated rats were sacrificed due to tumor within 3 weeks, six rats that had received 420 mg/m² BR96-DOX (12 mg/m² DOX) were free of detectable tumor at 7 weeks (Table 3). Rats were necropsied at 9 weeks postimplantation. At this time, five of the six rats were tumor free, and one of the rats had multiple lung tumors but no tumor elsewhere (Fig. 3).

In a repeat experiment, the long-term survival of rats bearing intrahepatic BN7005 tumors was evaluated (Fig. 4). Both untreated rats and rats treated with the MTD (12 mg/m²) of DOX were sacrificed at 21 days postimplantation due to extensive tumor burden. At necropsy, there was widespread tumor growth in liver and mesenteric lymph nodes and formation of hemorrhagic ascites. In contrast, at this time all six rats treated with 420 mg/m² BR96-DOX (12 mg/m² DOX) did not demonstrate any clinical signs of tumor burden, and when they were laparotomized at 3 weeks, three were completely free of detectable tumor and the other three had small tumors and only at the site of inoculation. The median survival time for BR96-DOX treated rats was 88 days; one of six rats was sacrificed at day 68 due to the presence of large liver tumors, and two of six rats died at day 88 of multiple lung metastases. Fifty % of the rats treated with BR96-DOX survived for the 26-week evaluation period, and necropsies performed at this time showed that these rats were free of detectable tumor.

Serum Antibodies to BR96-DOX Interfere with Its Therapeutic Efficacy; Treatment with DSG Counteracts Their Formation.

Because both the murine and human regions of the chimeric BR96 are foreign in rats, an antibody response against BR96-DOX was expected. Rats bearing s.c. BN7005 tumors were left as untreated controls, treated with 420 mg/m² IgG-DOX (12 mg/m² equivalent DOX) plus DSG, or treated with 420 mg/m² BR96-DOX (12 mg/m² equivalent DOX) with or without DSG during the first round of conjugate therapy. All drug treatments were initiated when tumors were approximately 500 mm³ in size, and treatments were administered every 4 days for a total of three injections. As shown in the Fig. 5, DSG did not interfere with the antitumor activity of BR96-DOX, which in fact was slightly better for rats that were treated with both BR96-DOX and DSG relative to that seen with BR96-DOX alone. As described above (Fig. 2 and Table 2), IgG-DOX was not active against established BN7005 tumors. Similarly, combined therapy with DSG and IgG-DOX treatment was not active against BN7005 tumors (Fig. 5). Of the BN7005-bearing rats treated with BR96-DOX alone, 4 of 5...
The BR96-DOX conjugate, BR96-DOX, was evaluated in several animal models, in which the effect of BR96-DOX treatment was assayed in an ELISA measuring specific binding of rat serum antibodies to chimeric BR96-DOX, expressed as A450/630 nm at a dilution of 1:100 (numbers in parentheses). These studies, which demonstrated that BR96-DOX was active against RCA colon carcinoma, evaluated in athymic mice was insensitive to unconjugated DOX administered at this dose. Importantly, in the BN7005 model of experimental liver metastasis, BR96-DOX administered at this dose produced 50% cures and 30% long-term responses, whereas unconjugated, optimized DOX was not active.

As expected, the BR96-DOX (chimeric mouse/human) immunoconjugate was immunogenic in rats. The development of anticonjugate antibodies resulted in reduced efficacy of BR96-DOX, but induction of anticonjugate Abs was decreased by cotreatment with DSG. These data are in agreement with observations made with immunoconjugates containing Pseudomonas exotoxin (18). The immunocompetent rat model may overpredict the problem with anticonjugate antibodies, because a mouse-human chimeric antibody may be more immunogenic in rats than in cancer patients. In fact, the production of clinically relevant anticonjugate antibodies has not been observed to date in the Phase I clinical trial of BR96-DOX (19).

These data demonstrate that MAb-directed targeting is also feasible when the tumor, like human cancers, arises in the species in which it is studied and is histocompatible with its host. CRs and cures of established s.c. RCA human and BN7005 rat colon tumors were obtained at tolerated doses of BR96-DOX although the rats, like humans, express the BR96-defined antigen in normal tissues of the gastrointestinal tract (8, 11). This indicates that the presence of normal tissue expression of the Lea antigen does not prevent tumor localization and/or result in unacceptable levels of toxicity of BR96-DOX, although no detailed investigation has been performed in cured rats on the possible subclinical damage of organs expressing Lea.

It is particularly encouraging that BR96-DOX could eradicate tumor nodules in the liver of immunocompetent rats, because this is a primary organ for naturally occurring metastases of colon carcinoma in humans.

Issues of tumor size, level of functional vasculature, antigenic heterogeneity, and inherent sensitivity or resistance to the targeted drug in the context of conjugate dose and schedule are discussed in a separate paper.

ACKNOWLEDGMENTS


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ANTITUMOR ACTIVITY OF BR96-DOX IMMUNOCONJUGATES


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