An Unmodified Anticarcinoma Antibody, BR96, Localizes to and Inhibits the Outgrowth of Human Tumors in Nude Mice

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ABSTRACT

The antitumor effects of an unmodified murine monoclonal antibody, BR96, were examined in nude mice bearing human lung adenocarcinoma xenografts. BR96, a murine IgG, that internalizes and is cytotoxic to cells expressing the antigen in vitro, also elicits strong antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity effector functions. Its in vivo antitumor effects were compared with those of its F(ab')2 fragments, a mouse-human chimeric form, and an IgG1 class switched variant of the original (IgG2) BR96. Antitumor effects were observed with antigen-positive tumor lines (but not with tumors which did not bind with BR96) and correlated with the levels of antigen expression as detected in vitro. The chimeric form of BR96 gave the strongest antitumor effects, followed by the murine IgG2, while limited effects were seen with the IgG1 and with F(ab')2 fragments of BR96, indicating that Fc-dependent host effector functions are primarily responsible for its in vivo activity. The antitumor effects observed were modest unless the antibody treatment was started on the day following tumor grafting.

INTRODUCTION

Many MAbs directed against tumor antigens have been studied in both animals and humans (1). Various methods have been devised to combine the ability of an unmodified MAb to “target” lesions with the anticancer activity of chemotherapeutic drugs or radioisotopes using the MAb as a transport vehicle rather than as an antitumor component. There is also an interest, however, in identifying MAbs with inherent antitumor activity in vitro, either alone or in combination with host components, and evaluating, in vivo, whether they can offer an attractive complement to existing tumor treatment strategies. This is particularly so, since MAbs capable of eliciting host effector functions offer the potential of less toxicity than may be possible when using immunoconjugates with chemotherapeutic agents or radioisotopes.

We report here in vivo studies with a MAb, BR96 (2), which was tested in the unmodified form for antitumor activity against human lung carcinomas xenografted into nude mice. BR96 is an IgG, that binds to a variant of a Le antigen expressed on most tumor cells from human carcinomas of colon, lung, breast, and ovary (2). The MAb can be internalized, and it is cytotoxic to a variety of antigen-positive tumor cell lines when tested by itself in vitro and is capable of eliciting ADCC and CDC effector functions (2). An isotype class switched (IgG1) variant of BR96 was also studied, as were a chimeric version of BR96 and F(ab')2 fragments derived from the murine BR96. Significant antitumor effects were seen with the murine IgG2 and chimeric versions of BR96, both of which mediate strong ADCC and CDC activities in vitro, while F(ab')2 fragments, which lack ADCC and CDC activities, had a much weaker, but still observable, antitumor effect in vivo.

MATERIALS AND METHODS

Monoclonal Antibodies. Our group has previously described (2) how BR96 was isolated as an IgG, from a hybridoma resulting from the fusion of spleen cells from a mouse immunized with a cell membrane preparation from a cultured human breast adenocarcinoma. Another MAb, BR64, which came from the same fusion and detected a related antigen (2), was used as a control in one experiment; like BR96, it can internalize. BR64 is a murine IgG, that lacks ADCC and CDC activity as well as cytotoxic activity by itself. IG5, which is a murine (IgG2) anti-Pseudomonas aeruginosa flagellar antibody (3) and does not bind to mammalian tissues, served as a control IgG, in most of our experiments. Chimeric 96.5 (4) was used as a nonspecific control in experiments with chimeric BR96. G19.4, which is an anti-CD3 (5) murine IgG1, was used as the control MAb in studies with a BR96 IgG1 switch variant (described below). F(ab')2 fragments of an anti-melanoma MAb, 96.5 (6), served as the nonbinding control in experiments with BR96 F(ab')2. All murine MAbs were purified from ascites, and all F(ab')2 fragments were prepared by standard methods (7).

Isotype Variants and Chimeric Antibodies of BR96 Origin. A hybridoma switch-variant-producing MAb of the IgG1 isotype was isolated using described procedures (8, 9). Chimeric BR96 (Chi BR96) was produced using a modification of a homologous recombination procedure which has been reported previously (4).

Tumor Lines. Cell lines H2707, H2981, and H2987 were developed at Bristol-Myers Squibb Pharmaceutical Research Institute in Seattle from human metastatic lung adenocarcinomas. Fluorescence-activated cell sorter analysis of cultured cells of these lines revealed high levels of BR96 binding to H2707 and H2987. The H2981 line did not bind BR96 and was used in experiments as an antigen-negative control line. (2) Histological examination of tissue sections prepared from tumors grown in vivo confirmed the fluorescence-activated cell sorter data by demonstrating the presence of the BR96-defined antigen on all lines except H2981. Subcutaneous implantation of 3.3 million lung carcinoma cells into nude mice resulted in palpable tumors approximately 8 days later.

Animal Model. Female nude Balb/c mice (nu/nu) (Harlan Sprague Dawley, Indianapolis, IN) received s.c. implantations in the rear flank of 10 million cells from one of the lung carcinoma cell lines (H2981, H2987, or H2707). Antibody treatment was initiated 24 h later (day 2) or on day 5 or day 8 postimplant. In each experiment, except when dose effects were examined, mice were given 1 mg MAb/injection (approximately 45 mg/kg); F(ab')2 fragments were given in 0.66-mg doses. Injections were given 3 days apart for a total of five injections. Using this schedule, which is based on the clearance kinetics of murine IgG in mice, a 3-day interval between the five injections resulted in exposure of the tumor to circulating MAb for over 2 weeks. In addition, the 3-day interval is less than the 6- to 7-day doubling time of the tumor lines in vivo. In the initial experiment, two additional 1-mg injections were given after the five injections. Treatment with control MAbs followed the same schedule and was always initiated on day 2.

Tumor volumes were calculated from the measurements of tumor...
length and perpendicular width by the formula

\[
\text{Tumor volume} = \frac{\text{Longest length} \times (\text{Perpendicular width})^2}{2}
\]

Significance between groups of treated mice was determined using the t test statistic for two means.

Localization Experiments. Localization studies were performed in mice carrying either H2707 or H2987 tumor xenografts. Radioiodinated intact BR96 of the IgG1 or IgG3, isotypes, ChiBR96, or Fab(α)2; fragments prepared from murine BR96 were administered with appropriate controls, at doses representative of those used in the therapy experiments with unmodified MAb. BR96 MAb were radioiodinated with 125I, while control MAb were radioiodinated with 131I using Iodogen (Pierce, Rockville, IL). Separation of labeled MAb from free iodine was performed using a G-25 Sephadex column. All iodinations were performed on the day of administration to the mice.

Mixtures of the specific and nonspecific MAb were administered simultaneously via the tail vein of each mouse, with each animal receiving approximately 5 μCi (185 kBq) of each radiolabeled MAb. At selected times mice were anesthetized, bled through the orbital plexus, and sacrificed. Selected tissues were removed, weighed, and counted in a dual-channel gamma counter capable of differentiating between the two iodine isotopes. Selected tissues included blood, tumor, liver, spleen, kidney, lungs, and thigh.

The corresponding cpm in each organ was analyzed using a computer program to correct for 125I Compton scatter into the 131I window. Correction for 131I decay was performed by counting a 0.2 μl aliquot of the injected dose with each set of tissue samples. Calculation of the activity in the blood was estimated assuming that the blood volume of a mouse is equal to 8% of its body weight. Distribution of specific and nonspecific MAb was expressed by percentage injected dose, calculated by cpm in organ/cpm injected × 100%. The percentage injected dose was then used to calculate the percentage injected dose/g tissue.

RESULTS

Experiments were initially done in which treatment of tumor-bearing mice with unlabeled MAb BR96 (IgG3) was started 24 h postimplant and was followed by six more injections on days 5, 8, 11, 14, 19, and 21 postimplant. MAb BR64 was used as a control and was given to the animals at the same dose and time points. As seen in Fig. 1 significant antitumor effects were observed following treatment with BR96 (P < 0.005, P < 0.0005, P < 0.0005, days 8, 21, 28 postimplant, respectively), while there were few if any antitumor effects with BR64 as compared to a group given PBS only. No toxicity was apparent in any group. Complete regressions occurred in 2 of 10 animals receiving BR96, and these animals remained tumor-free for the duration of the experiment. Tumors in the remaining 8 mice grew similarly to tumors in mice treated with BR64 or PBS, except for an initial inhibition.

We then examined the antitumor activity of BR96 (IgG1) against staged tumors. Experiments were performed with the antigen-positive H2987 and H2707 lung carcinoma lines. Mice were implanted with approximately 3 times as many cells as necessary to establish palpable tumors by 8 days, and treatment was started either 5 or 8 days post tumor implant, when all mice had palpable tumors in the range of 75-100 mm3. The administration of MAb was repeated five times 3 days apart. Fig. 2 shows data from H2987 xenografts, and Fig. 3 gives results from H2707. While staged tumors were much less responsive to BR96, significant effects were apparent in the form of a delay in tumor growth (P < 0.05, day 32 postimplant of H2987, and P < 0.05, day 29 postimplant of H2707 for each BR96-treated group compared to PBS). There were few if any differences in mean tumor volumes, depending on whether treatment was initiated on day 5 or day 8 postimplant. However, variations in the responses of individual tumors were observed at the end of treatment. Thus 9 of 10 mice bearing H2707 xenografts were tumor-free when the treatment had started on day 2, as compared to 5 of 10 mice and 3 of 10 mice when treatment was started on days 5 and 8, respectively. Analogous but less impressive effects were observed with H2987. The tumor that was present in 1 of the 10 mice carrying H2707 and treated from day 2 was excised, and its cells were suspended.
INHIBITION OF HUMAN TUMORS IN NUDE MICE BY MAb BR96

DAYS POST IMPLANT

Fig. 4. Mean volumes of tumors (±SEM) from mice (8/group) receiving i.v. injections with a range of BR96 (IgG3) doses on five occasions 3 days apart beginning on day 2 and as indicated by arrows after s.c. implantation of 10^7 H2707 cells. IgG5 (IgG3) was injected i.v. 1 mg/injection. on the same days. Control mice were given an equal volume of PBS beginning on day 2.

DAYS POST IMPLANT

Fig. 5. Mean volumes of tumors (±SEM) from mice (8/group) receiving injections (1 mg/injection) of the IgG1 isotype switch variant of BR96, BR96 (IgG3), or BR64 (IgG3) on five occasions 3 days apart beginning on day 2 and as indicated by arrows after s.c. implantation of 10^7 H2707 cells. Control mice were given equal volumes of PBS

and immediately assayed for the presence of the BR96 antigen by fluorescence-activated cell sorter analysis with fluorescein isothiocyanate-labeled BR96. Most of the intact cells recovered from this tumor still strongly expressed the antigen to which BR96 could bind, suggesting that growth of this tumor was not due to an antigen-negative clone selected for by BR96 treatment.

Dose effects were examined by reducing the injected amount of murine IgG3 in half-log increments from 1 to 0.032 mg/injection given to mice bearing H2707 xenografts. The treatment schedule remained five times, 3 days apart, beginning on day 2. Fig. 4 demonstrates that the antitumor effects decreased as the dose was lowered, although the difference between the 0.32-mg and 0.1-mg doses was small. To further explore the mechanisms responsible for the antitumor effects observed when animals were treated with BR96, the activities of an isotype switched mouse variant, BR96 IgG1, a mouse-human chimeric version of BR96, and F(ab')2 fragments prepared from BR96 were also tested against the H2707 tumor line. The mean tumor volumes for groups of mice treated with the IgG3 variant of BR96 (1 mg/dose) are shown in Fig. 5. The IgG3 variant had no more antitumor effect than the BR64 control MAb, both of which were slightly better than the PBS control. However, the chimeric version of BR96 (0.32 mg/dose) resulted in antitumor effects at least as good as and apparently exceeding those of the murine IgG3, BR96, as shown in Fig. 6.

The effects of the IgG1 dose and MAb form were most pronounced in the number of mice without palpable H2707 tumors immediately after treatment, 15 days post tumor implant. All mice that received BR96 (IgG3) at the 1 mg/dose were without palpable tumors at the end of treatment. The number of mice without palpable tumors at that time decreased as the IgG3 dose was lowered. Once treatment stopped the tumors began to grow rapidly. There were no apparent toxic effects in any treated animal. Surprisingly, in view of the data with the IgG3 switch variant, 3 of the 8 mice which received the one dose tested of F(ab')2 were without palpable tumors at the end of treatment. Treatment with 0.32 mg/dose of the chimeric BR96 resulted in 6 tumor-free mice compared to 4 mice of 8 in the group that received an equal amount of the IgG3. There were no dose effects observed in the mice that received the similar range of doses of the IgG1 switch variant. Mice treated with PBS or the nonspecific MAbs all had palpable tumors at the end of treatment, 15 days postimplant. In another experiment, increasing the dose to 2 mg/injection did not result in greater antitumor activity (data not shown).

The antitumor capabilities of BR96 were also examined against a tumor which does not express the BR96 antigen, H2981. Mice were implanted s.c. with 10 million tumor cells, and treatment was started on day 2, with each mouse receiving 1 mg of MAb once every 3 days for five injections. Fig. 7 demonstrates that BR96 (IgG3) had no effect on the H2981 line.

Localization Experiments. The ability of radiolabeled BR96

DAYS POST IMPLANT

Fig. 6. Mean volumes of tumors (±SEM) from mice (8/group) injected with BR96 F(ab')2 fragments, the chimeric version of BR96, IgG5 (IgG3), and 2 doses of BR96 (IgG3) beginning on day 2 and as indicated by arrows after s.c. implantation of 10^7 H2707 cells. Control mice received injections of equal volumes of PBS.

DAYS POST IMPLANT

Fig. 7. Mean volumes of tumors (±SEM) from mice (8/group) injected i.v. with 1 mg/dose of BR96 (IgG3) or with an equal volume of PBS beginning on day 2 and as indicated by arrows after s.c. implantation of 10^7 H2981 cells.

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to localize to tumor sites was examined in mice transplanted with either the H2987 or the H2707 carcinoma lines, using radiolabeled antibodies as probes. All mice had palpable tumors approximately 2 weeks earlier. Table 1 summarizes the biodistribution resulting from the s.c. implantation of 10 million cells approximately 2 weeks earlier. Table 1 summarizes the biodistribution experiments.

### Table 1. Summary of biodistribution experiments

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<th>MAb</th>
<th>Dose (mg)</th>
<th>Tumor</th>
<th>Blood</th>
<th>Tumor</th>
<th>Liver</th>
<th>Spleen</th>
<th>Kidney</th>
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<td>2.2</td>
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<td>1.6</td>
<td>2.4</td>
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<td>1.8</td>
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<td>2.8</td>
</tr>
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<td>13.4</td>
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<td>1.8</td>
<td>1.6</td>
<td>1.9</td>
<td>3.5</td>
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<tr>
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<td>H2707</td>
<td>&lt;0.25</td>
<td>&lt;0.25</td>
<td>&lt;0.25</td>
<td>&lt;0.25</td>
<td>&lt;0.25</td>
<td>&lt;0.25</td>
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<tr>
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<td></td>
<td></td>
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<tr>
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<td>0.65</td>
<td></td>
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The highest tumor:blood uptake ratio was seen with the chimeric BR96, although the specific:nonspecific ratio in the tumor was lower than with the other forms of BR96 tested. The tumor:blood ratios for the two murine isotypes were practically identical. This is noteworthy in view of the differences in therapeutic efficacy of the various forms of unlabeled MAb.

The portion of the dose detected in a particular organ was relatively constant over the dose range used. It may therefore be possible to increase the amount of MAb in the tumor by increasing the dose. F(ab')2 fragments were cleared more rapidly than intact antibodies and had poor uptake in tumors.

### DISCUSSION

We have shown that MAb BR96 has significant antitumor effects when tested, in the unmodified form, in nude mice xenografted with human lung adenocarcinoma. The antigen to which BR96 can bind must be expressed in vivo for these effects to occur, inasmuch as there was no inhibition of a lung adenocarcinoma lacking the BR96-defined antigen. Consistent with previous in vitro data (2), mice with a higher antigen-expressing tumor line, H2707, displayed greater antitumor effects than mice grafted with line H2987, which expressed less antigen. Our findings are similar to those obtained with certain other MAb (10-13), including antibodies which can have antitumor activity of their own, the most notable of which being an antibody against a neu oncogene product (14). BR96 differs from the latter antibody in its much broader tumor specificity, however.

The antitumor effects observed with BR96 may have occurred through several different mechanisms. While it is difficult to separate these mechanisms in the nude mouse model, our data suggest that direct killing by BR96 is not a major component against staged tumors, since only limited antitumor effects were seen with BR96 F(ab')2 fragments, which by lacking the Fc portion of the molecule could only have acted directly on the tumor cells and not by involving complement or effector cells. Since the F(ab')2 fragments were cleared rapidly, it is possible, however, that antitumor effects would have been greater had the fragments remained longer in the animal. The IgG1 murine switch variant has a less cytotoxic effect in vitro than any of the other versions of BR96 tested, including F(ab')2 fragments, and does not elicit ADCC or CDC. While it localized to the tumor in a manner similar to that of the murine IgG1 isotype, its antitumor activity in vivo was much less than those of the murine IgG1 and chimeric BR96 and even less than that of the F(ab')2 fragments.

With the exception of the F(ab')2 fragments, each of the murine isotypes and the chimeric version of BR96 localized at the tumor in comparable amounts. Therefore, the antitumor effects observed in our model were most likely to be due to the elicitation of effector functions such as ADCC or CDC (15, 16), with ADCC probably playing a larger role than CDC, judging from observations made in other systems (15, 17). Chimeric mouse-human IgG1 MAb have been shown to mediate highly effective ADCC (18–20), and murine MAb of the IgG1 isotype can often elicit strong ADCC (15). The data reported in this paper are consistent with these observations: the strongest antitumor effects were seen with chimeric BR96 followed by the IgG1 version, while the BR96 IgG2 variant and the F(ab')2 fragments, which do not elicit ADCC or CDC (2) in vitro, had only limited antitumor activity in the mice.

The strongest antitumor effects were seen when treatment was initiated within 2 days after tumor cell implantation. At this time, the tumor would still be very small and should allow easier access, not only for the MAb molecules and complement, but also for effector cells such as natural killer cells and macrophages. Since no toxicity was observed in the mice, it should be possible to continue treatment for a much longer period of time. Additionally, the antitumor effects of unmodified BR96 might enhance the efficacy of other treatment modalities added concomitantly.

One may speculate that the unmodified chimeric and murine IgG1 versions of BR96 may also have therapeutic activity in humans (e.g., toward micrometastases), although it is hard to predict to what extent tumor cells would be destroyed without causing unacceptable damage to those subpopulations of normal cells of the gastrointestinal tract to which BR96 binds (2). The fact that only modest effects were observed in nude mice with staged tumors suggests, however, that unmodified BR96 will be much less effective for therapy than BR96 to which an antitumor agent such as a drug or radioisotope has been coupled, unless procedures can be developed for increasing the ability of BR96 to kill tumor cells in vivo via ADCC and/or CDC.

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### REFERENCES

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