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 IgG₁ 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 vitro antitumor effects were compared with those of its F(ab’₂)₂ fragments, a mouse-human chimeric form, and an IgG₁ class switched variant of the original (IgG₁) 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 IgG₁, while limited effects were seen with the IgG₂ and with F(ab’₂)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 antitumor 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 immunomonomers 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 IgG₁, that binds to a variant of a Le¹ 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 (IgG₂) variant of BR96 was also studied, as were a chimeric version of BR96 and F(ab’₂)₂ fragments derived from the murine BR96. Significant antitumor effects were seen with the murine IgG₁ and chimeric versions of BR96, both of which mediate strong ADCC and CDC activities in vitro, while F(ab’₂)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 IgG₁ 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 IgG₁ that lacks ADCC and CDC activity as well as cytotoxic activity by itself. IIG5, which is a murine IgG₂ anti-Pseudomonas aeruginosa flagellar antibody (3) and does not bind to mammalian tissues, served as a control IgG₁ 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 IgG₂, which is a murine (IgG₂) anti-Pseudomonas aeruginosa flagellar antibody (3) and does not bind to mammalian tissues, served as a control IgG₂ 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 IgG₂, which is a murine (IgG₂) anti-Pseudomonas aeruginosa flagellar antibody (3) and does not bind to mammalian tissues, served as a control IgG₂ 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 IgG₂, which is a murine (IgG₂) anti-Pseudomonas aeruginosa flagellar antibody (3) and does not bind to mammalian tissues, served as a control IgG₂ 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 IgG₂, which is a murine (IgG₂) anti-Pseudomonas aeruginosa flagellar antibody (3) and does not bind to mammalian tissues, served as a control IgG₂ 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 IgG₂, which is a murine (IgG₂) anti-Pseudomonas aeruginosa flagellar antibody (3) and does not bind to mammalian tissues, served as a control IgG₂ 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 IgG₂, which is a murine (IgG₂) anti-Pseudomonas aeruginosa flagellar antibody (3) and does not bind to mammalian tissues, served as a control IgG₂ 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 IgG₂, which is a murine (IgG₂) anti-Pseudomonas aeruginosa flagellar antibody (3) and does not bind to mammalian tissues, served as a control IgG₂ 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 IgG₂, which is a murine (IgG₂) anti-Pseudomonas aeruginosa flagellar antibody (3) and does not bind to mammalian tissues, served as a control IgG₂ 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 IgG₂, which is a murine (IgG₂) anti-Pseudomonas aeruginosa flagellar antibody (3) and does not bind to mammalian tissues, served as a control IgG₂ 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 IgG₂, which is a murine (IgG₂) anti-Pseudomonas aeruginosa flagellar antibody (3) and does not bind to mammalian tissues, served as a control IgG₂ 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 IgG₂, which is a murine (IgG₂) anti-Pseudomonas aeruginosa flagellar antibody (3) and does not bind to mammalian tissues, served as a control IgG₂ in most of our experiments.

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)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...
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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. Radiiodinated intact BR96 of the IgG\(_1\) or IgG\(_2\) isotypes, ChiBR96, or F(ab)\(_2\) fragments prepared from murine BR96 were administered with appropriate controls, at doses representative of those used in the therapy experiments with unmodified Mabs. BR96 Mabs were radiiodinated with \(^{125}\)I, while control Mabs were radiiodinated with \(^{131}\)I 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 Mabs were administered simultaneously via the tail vein of each mouse, with each animal receiving approximately 5 \(\mu\)Ci (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 \(^{131}\)I Compton scatter into the \(^{125}\)I window. Correction for \(^{131}\)I decay was performed by counting a 10-\(\mu\)l 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 Mabs was expressed by percentage injected dose, calculated by cpm in organ/cpm injected \(\times 100\%\). 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 (IgG\(_1\)) was started 24 h postimplant and was followed by 6 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 (\(P < 0.0005\), \(P < 0.0005\), 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 (IgG\(_1\)) 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 mm\(^3\). The administration of Mab was repeated five times 3 days apart, and staging continued. 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 for further analysis.

Fig. 1. Mean volumes of tumors (±SEM) from mice (10/group) implanted with 10^7 H2987 cells s.c. Mice were given i.v. injections of murine BR96 (IgG\(_1\)) or BR64 (IgG\(_1\)) the following day (day 2) and as indicated by arrows, with each mouse receiving 1 mg MAb/injection. Control mice received an equal volume of PBS.

Fig. 2. Mean volumes of tumors (±SEM) from mice (10/group) receiving injections on five occasions 3 days apart. Mice were given BR96 (IgG\(_1\)) beginning on day 2, 5, or 8 after s.c. implantation of 10^7 H2987 cells. IgG5 (IgG\(_1\)) was given (five injections 3 days apart) beginning on day 2. Control mice were given an equal volume of PBS (five injections 3 days apart) beginning on day 2.

Fig. 3. Mean volumes of tumors (±SEM) from mice (10 mice/group) receiving injections on five occasions 3 days apart. Mice were given BR96 (IgG\(_1\)) beginning on day 2, 5, or 8 after s.c. implantation of 10^7 H2707 cells. IgG5 (IgG\(_1\)) was given (five injections 3 days apart) beginning on day 2. Control mice were given an equal volume of PBS (five injections 3 days apart) beginning on day 2.
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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.

The effects of the IgG3 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 IgG1 switch variant, 3 of the 8 mice which received the one dose 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 MAb 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 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.

Fig. 5. Mean volumes of tumors (±SEM) from mice (8/group) receiving injections (1 mg/injection) of the IgG3 isotype switch variant of BR96, BR96 (IgG3), or BR64 (IgG1) 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.

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.

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 in tumor as compared to blood and several organs. As shown in the table, the largest differences between specific and nonspecific MAb uptake occurred in tumors. There was a difference in blood concentrations of BR96 IgG, and the nonspecific uptake was greater in the murine carcinoma 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. The 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 MAbs (10-13), including antibodies which can have antitumor activity in vivo, 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 might 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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