Divergent Effects of 4-1BB Antibodies on Antitumor Immunity and on Tumor-reactive T-Cell Generation

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ABSTRACT

4-1BB is an inducible receptor-like protein expressed rapidly by both CD4 and CD8 T-cells after activation. 4-1BB cross-linking, either by binding to 4-1BBL or by antibody ligation, delivers a costimulatory signal to enhance T-cell activation and proliferation. Previous studies have demonstrated that the administration of 4-1BB monoclonal antibodies (mAbs) induces antitumor immune responses. In the current study using several murine tumors, we examined the systemic effects of 4-1BB mAb on the growth of s.c., intracranial, and pulmonary metastases. In addition, the effects of 4-1BB mAb on the generation of antitumor effector T cells were examined. Treatment of 3-day i.c. MCA 205 sarcoma and GL261 glioma with the antibody resulted in prolongation of survival and cure of disease in some mice, whereas only minimal therapeutic effects were observed in established s.c. and pulmonary tumors. No antitumor effects against the poorly immunogenic B16/D5 melanoma were observed. Interestingly, successful treatment of i.c. tumors induced concomitant regression of s.c. tumors. Experiments using severe combined immunodeficient mice and mice depleted of either CD4 or CD8 T cells demonstrated T-cell dependence of the antitumor effects. For generation of effector T cells in the tumor-draining lymph nodes (LN), administration of 4-1BB mAb had adverse effects, despite the apparent hypersensitivity of the LN. During in vitro activation of tumor-draining LN T cells with anti-CD3 and interleukin 2, the 4-1BB mAb augmented proliferation, resulting in an increase in CD8 T cells. However, they were less therapeutic than not treated LN cells. In adoptive immunotherapy, the coadministration of 4-1BB mAb enhanced the therapeutic efficacy. These results thus demonstrate the limits and potential advantages of 4-1BB antibody interactions with antitumor T cells in vivo and in vitro and suggest that therapeutic interactions of the antibody may be used in a variety of immunotherapeutic approaches.

INTRODUCTION

4-1BB is an inducible protein expressed on the surface of activated T lymphocytes that has been shown to exhibit important regulatory activity in the development of immune responses. It is a member of the tumor necrosis factor/nerve growth factor family of receptors that is distinctly different from CD28-mediated costimulation. It has been shown that in vivo administration of 4-1BB antibodies results in the regression of established s.c. and pulmonary P815 mastocytoma and AG104A sarcoma (4). The antitumor effect was dependent upon participation of both CD4 and CD8 T cells, and systemic immunity was induced subsequently.

The use of mAbs that bind to T-cell costimulatory molecules to enhance antitumor immune responses has shown mixed results, depending upon the immunogenicity of the tumor and animal strain (5–7). Although several reports of using mAbs that stimulate the CD28 pathway showed significant tumor regressions in vivo, the application of this strategy to poorly immunogenic tumors was generally ineffective. This brings into question the importance of the tumor type, anatomical site, and degree of T-cell activation in vivo for the evaluation of therapies targeted toward costimulation of antigen-specific T cells. The purpose of this study was to evaluate antitumor activities enhanced through costimulation of anti-4-1BB. In the past, we identified that LNs draining a progressive tumor contained specific tumor-sensitized T cells. Upon in vitro stimulation and expansion with anti-CD3 and IL-2, these cells generated potent therapeutic reactivity when systemically transferred to tumor-bearing mice (8). Therefore, we also evaluated the effect of anti-4-1BB on the in vivo sensitization, ex vivo activation, and effector phases of cells from LNs draining a progressively growing tumor. The results of this study suggest that the efficacy of antigen-specific costimulation via 4-1BB is multifaceted and may be exploited for therapeutic benefits.

MATERIALS AND METHODS

Experimental Animals. Female C57BL/6 (B6) mice, 6–8 weeks of age, were purchased from the Biological Testing Branch, National Cancer Institute (Frederick, MD). Mice were maintained under pathogen-free conditions and used for experiments at ages 8–12 weeks. B6 SCID (Plkdr^−/−) mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

Tumors. The 3-methylcholanthrene-induced fibrosarcoma MCA 205 is a weakly immunogenic tumor of B6 origin and was passaged s.c. (9). Solid s.c. tumors were harvested, digested in 0.01% Dnase, 0.1% collagenase, and 2.5 units/ml hyaluronidase (Sigma Chemical, St. Louis, MO) for 2–4 h at room temperature and passed through a 100 μm filter for single-cell suspensions. Cells were washed twice in HBSS and resuspended in HBSS at desired concentrations. Tumors were used between passages 5 and 8 to reduce genetic drift from the original tumor line.

B16/D5 is a poorly immunogenic subclone of the B16/BL6 melanoma that has been passaged in tissue culture (10). MC38 is a dimethylylazazine-induced colon adenocarcinoma of B6 origin obtained from the laboratory of S. A. Rosenberg (NIH, Bethesda, MD; Ref. 11). The GL261 tumor, a 3-methylcholanthrene-induced glioma syngeneic to B6 mice, was obtained from the Division of Cancer Treatment Repository (Frederick, MD; Ref. 12). All cell lines were maintained and passaged in vitro in CM consisting of RPMI 1640 with 10% FCS, 2 mM l-glutamine, antibiotics, and antifungal agents (Life Technologies, Inc., Grand Island, NY) at 37°C and 5% CO₂. Cells were harvested by brief treatment with 0.25% trypsin/0.02% EDTA (Life Technologies, Inc.) and resuspended in HBSS for animal inoculations.
Tumor Inoculations. All animal injections and procedures were performed according to institutional animal protocols. i.c. tumors were initiated in B6 mice anesthetized with 1 mg of nembutal i.p. Routinely, \(1 \times 10^7\) tumor cells in 10 \(\mu\)l of HBSS were delivered into the right parietal lobe using a shielded 27-gauge needle at a depth of 4 mm, as previously described (13). This injection technique resulted in establishment of i.c. tumors with virtually no immediate mortality.

Pulmonary tumors were established by i.v. tail vein injection of tumor cells suspended in 1.0 ml of HBSS. On days 17–20 after injection, mice were sacrificed, and lungs were harvested and counterstained with India ink as described (14). Pulmonary tumors on the surface of the lung were then enumerated after bleaching in Fekete’s solution.

s.c. tumors were established in the flank by inoculation of tumor cells in 50 –100 \(\mu\)l of HBSS using a 27-gauge needle. Tumors were measured every 3– 4 days with a Vernier caliper. Perpendicular measurements were obtained and reported as tumor area in \(\text{mm}^2\).

Generation of Tumor-reactive T Cells and Adoptive Immunotherapy. Tumor-reactive T cells were generated from tumor-draining LNs, as described previously (15–17). Briefly, B6 mice were inoculated s.c. with 1.5 \(\times\) \(10^6\) MCA 205 tumor cells in both flanks. After 12 days of tumor growth, draining inguinal LNs were surgically obtained, and single cells were prepared and activated in CM on 24-well plates (Costar, Cambridge, MA.) with immobilized anti-CD3 mAb (2C11) at a density of \(4 \times 10^6\) cells/2 ml/well for 48 h. After activation, cells were harvested and expanded in gas-permeable culture bags (Baxter Fenwal, Deerfield, IL) in CM with 4 units/ml IL-2 for 72 h. Finally, these cells were resuspended in HBSS and adoptively transferred in 1 ml i.v. via the tail vein to mice bearing 3-day established pulmonary metastases. Attempts were also made to treat 10-day established tumors. In this case, all tumor-bearing mice were sublethally irradiated (500 rads; Cesium source) before receiving activated cells. At 17–20 days after tumor growth, mice were euthanized, and pulmonary tumors were enumerated. The number of tumors was reported as mean \(\pm\) SE for each group of five mice.

Fig. 1. Lack of significant therapeutic activity of 4-1BB mAbs against s.c. tumors. Mice bearing 3-day established s.c. tumors of MCA 205 (A), B16/D5 (B), or MC38 (C) were treated on days 3 and 6 with 4-1BB mAb 3E1, 100 \(\mu\)g/ml i.p. or 300 \(\mu\)g i.p. (A, Expt. 3). Data represent average tumor areas of five mice per group; bars, SE. Numbers in parentheses are \(\mu\)g of mAb administered. In MCA 205 Expt. 3, although tumor growth in mAb-treated mice is significantly suppressed, there is no difference between mice treated with 100 and 300 \(\mu\)g of 4-1BB mAb.

**RESULTS**

Therapeutic Effects of 4-1BB mAb on Established Tumors. In an attempt to determine the therapeutic effects of 4-1BB mAb for the treatment of experimental metastases established at distinct anatomical sites, the first set of experiments focused on treating animals with 3-day established s.c. metastases derived from three syngeneic tumors of various immunogenicities (Fig. 1). Antibody administered at doses of 100 \(\mu\)g i.p. on days 3 and 6 after s.c. inoculation did not demonstrate significant antitumor reactivity (Fig. 1A). Increasing doses to 300 \(\mu\)g on day 6 of treatment also failed to show significant tumor suppression (Fig. 1A, Expt. 3). Thus, for the three murine tumors, s.c.

mAbs. 1D8 and 3E1 anti-4-1BB mAbs, both of which are of rat origin and bind and activate murine 4-1BB receptor, were generously supplied by Bristol-Myers Squibb (Seattle, WA). In previous studies, these two mAbs have shown virtually identical therapeutic effects (4). RlG was used as control (PharMingen, San Diego, CA). Antibodies were administered i.p in 0.5 ml of HBSS.

**In Vivo T-Cell Depletion.** CD4 and CD8 depletion of mice was performed by administering anti-CD4 (GK1.5) and anti-CD8 (2.43) mAbs as described previously (18). Mice were injected with 0.2 ml ascites diluted to 1 ml of HBSS i.v. via the tail vein on day –1 and day 6 after tumor inoculation. Analyses of the spleen cells obtained 7 days after the last mAb administration confirmed the efficacy of the respective T-cell depletion of \(>95\%\).

**FACS Analysis.** The following FITC- and phycoerythrin-conjugated antibodies were used to analyze tumor-draining LNs: anti-Thy 1.2, CD4, CD8, CD25, and L-selectin (PharMingen). Samples were analyzed using FACSCalibur (Becton Dickinson, Mountain View, CA).

**Statistical Analysis.** Krukis-Wallis and Wilcoxon rank-sum tests were used for nonparametric analyses. Tested variables were deemed significant at \(P < 0.05\).

**Fig. 1.** Lack of significant therapeutic activity of 4-1BB mAbs against s.c. tumors. Mice bearing 3-day established s.c. tumors of MCA 205 (A), B16/D5 (B), or MC38 (C) were treated on days 3 and 6 with 4-1BB mAb 3E1, 100 \(\mu\)g/ml i.p. or 300 \(\mu\)g i.p. (A, Expt. 3). Data represent average tumor areas of five mice per group; bars, SE. Numbers in parentheses are \(\mu\)g of mAb administered. In MCA 205 Expt. 3, although tumor growth in mAb-treated mice is significantly suppressed, there is no difference between mice treated with 100 and 300 \(\mu\)g of 4-1BB mAb.
tumors did not respond to this regimen of antibody treatment. For the treatment of pulmonary metastases established 3 days after i.v. inoculation of mice with tumor cells, an identical regimen was tested with 100 and 300 \( \text{mg} \) on days 3 and 6. Although no significant reduction of the number of pulmonary tumors was observed with 100 \( \text{mg} \) of mAb, increasing doses to 300 \( \text{mg} \) demonstrated significant antitumor effects \((P < 0.05)\) against the MCA 205 tumor (Fig. 2). The similar high dose antibody treatment did not affect the poorly immunogenic B16/D5 melanoma. For the MC38 tumor, although there seemed to be a reduction of the number of pulmonary metastases in mice treated with the mAb, there was no difference when compared with the control RlG-treated mice.

The effects of 4-1BB mAb were finally tested for the treatment of experimental i.c. tumors. B6 mice were inoculated i.c. with various numbers of different tumor cells so that all animals would succumb to the growing i.c. tumors at 17–25 days. Groups of tumor-bearing mice were treated on days 3 and 6 with 100 \( \mu \text{g} \) of antibodies. As shown in Fig. 3, for animals harboring MCA 205 sarcoma or GL261 glioma, treatment with mAb resulted in significant survival advantages \((P < 0.05)\). In fact, four and two of the groups of five mice with MCA 205 tumor treated with 3E1 and 1D8 mAb were apparently cured of the disease, respectively. For the GL261 glioma, prolongation of survival and cure of two of five mice were observed after mAb treatment. Again, no antitumor effects of the antibody were seen for the treatment of the poorly immunogenic B16/D5 tumor. Taken together, the therapeutic effects of 4-1BB antibodies depended on not only the immunogenicity of the experimental tumor but also on the anatomical site of tumor growth.

### Phenotype Analysis of T Cells Involved in 4-1BB mAb-mediated Tumor Regression

Previous studies suggested that both CD4 and CD8 T cells were required for the 4-1BB-induced tumor immunity, although the antibody seemed to preferentially induce more CD8 T-cell proliferation (4, 19). Our approach to analyze the T-cell involvement started by using SCID mice as the recipient for 4-1BB mAb therapy. For the treatment of MCA 205 i.c. tumors, the survival advantage was not evident in SCID mice, whereas administration of 1D8 antibodies improved survival in immunocompetent mice (Fig. 4A). To evaluate the relative role of T-cell subsets in vivo, both CD4 and CD8 T cells were required for tumor rejection because depletion of either CD4 or CD8 T cells abrogated the antitumor effects of the antibody (Fig. 4, B and C).

### Systemic Antitumor Immunity Induced by the Antibody-mediated i.c. Tumor Eradication

Mice cured of MCA 205 i.c. tumor by the 4-1BB antibody treatment were challenged s.c. with either MCA 205 or B16/D5 tumors. Although all seven mice rejected the second challenge with 1.5 \( \times 10^6 \) MCA 205 tumor cells, none of the five cured mice were protected by lethal challenge (1 \( \times 10^5 \)) with B16/D5 tumor cells (data not shown). To examine the potency of this specific immunity, we inoculated B6 mice with MCA 205 tumor cells, both i.c. and s.c. simultaneously on the same day before 4-1BB mAb treatment commenced on days 3 and 6. In this type of experimental setting, untreated mice with both i.c. and s.c. tumors usually succumb.

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**Fig. 2.** Minimal therapeutic activity of 4-1BB mAbs against pulmonary tumors. Mice bearing 3-day established pulmonary tumors were treated with 4-1BB mAbs on days 3 and 6 at the doses indicated. Lungs were harvested 17 days after tumor inoculation, and metastases were enumerated. Mice with pulmonary tumors “too numerous to count” were given a value of 250. Mice with the MCA 205 tumor treated with 300-\( \mu \text{g} \) doses of mAb show a significant reduction of the numbers of tumor nodules compared with that from mice treated with RlG \((P < 0.05)\).

**Fig. 3.** 4-1BB mAb improves survival in mice bearing MCA 205 and GL261 but not B16/D5 i.c. tumors. Mice bearing 3-day established i.c. tumors were treated with either 4-1BB mAbs 3E1 or 1D8, 100 \( \mu \text{g} \) i.p. on days 3 and 6. Survival was significantly improved over control RlG-treated mice (MCA 205) or HBSS-treated mice (GL261) in mice treated with 4-1BB mAb \((P < 0.05)\).
to the growth of the i.c. tumors, although the s.c. tumors also grew progressively. Mice with either i.c. tumor alone or both i.c. and s.c. tumors responded to the antibody treatment, as evident by prolongation of survival and cure of some mice (Fig. 5A). Monitoring the growth of s.c. tumors revealed that treatment of i.c. tumors with the antibody induced sufficient antitumor immunity to suppress the growth of the concomitant s.c. tumors (Fig. 5B). Because some mice with both tumors died on days 27–30 because of progression of the i.c. tumor, the sizes of the s.c. tumors were measured and reported up to the time of death. Treatment of the animals bearing only s.c. tumors with the antibody resulted in the retardation of tumor growth, but all animals eventually had progressive tumors. The rejection of a simultaneous s.c. tumor during rejection of the i.c. tumor suggested that the development of a systemic antitumor immunity was crucial for the antibody-mediated therapeutic effects.

**Effects of 4-1BB mAb Administration on the Tumor-immune T-Cell Generation in Tumor-draining LNs.** The observation of T cell-dependent antitumor effects of 4-1BB mAb prompted us to examine the effects of the antibody in the sensitization of T cells in tumor-draining LNs. In numerous previous studies, we demonstrated tumor-draining LNs to be a reliable source rich in tumor-sensitized T cells (8). Upon activation and expansion in vitro, these LN T cells mediated potent antitumor effects when systemically transferred to tumor-bearing mice (15–17). Mice inoculated s.c. with MCA 205 tumor cells were treated with injections of 100 μg of 4-1BB mAb i.p. R1g served as a control. On day 12 of tumor growth, draining LNs were harvested, and cells were activated according to the anti-CD3/IL-2 method described previously (15). Analysis of the T-cell phenotypes before and after activation revealed that there were little differences between LN cells from 4-1BB mAb-treated and control mice (Fig. 6), although anti-4-1BB-treated mice had significantly higher numbers of cells per LN than that of control mice (22 ± 6 × 10^6/LN versus 11.6 ± 6 × 10^6/LN; P < 0.01; average of four experiments). The activated LN cells were tested for their in vivo antitumor effects by adoptive transfer into mice with 3-day established MCA 205 pulmonary tumors. The results of the survival of the mice are depicted in Fig. 6. Mice that received 10 × 10^6 control LN cells demonstrated prolonged survival, and 50% of the treated mice were cured, whereas the transfer of 3 × 10^6 cells had no therapeutic benefit. By contrast, mice that received 10 × 10^6 LN cells from 4-1BB mAb-treated animals did not show therapeutic effects. In fact, they succumbed to the growing tumors with a median survival time of 44 days, which is shorter than the survival time (median, 54 days) of not treated mice. Taken together, these results indicate that despite its ability to induce a systemic antitumor immunity, the 4-1BB antibody suppressed the transient local immune response in the tumor-draining LNs.

The ligation of 4-1BB costimulation on T cells enhances their functional and proliferative activities (3, 4). During the in vitro anti-CD3/IL-2 activation, the addition of 10 μg/ml 4-1BB mAb resulted in increased cell proliferation from 4.2 ± 0.1-fold in untreated culture to 10.6 ± 0.7 (P < 0.05). Consistent with results published previously, there was an increased proportion of CD8 cells (84%) with

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**Fig. 4.** Antitumor activity of 4-1BB mAb is dependent on both CD4 and CD8 T cells. Mice bearing 3-day established MCA 205 i.c. tumors were treated with 4-1BB mAb as described in Fig. 3. Survival advantage of groups treated with 4-1BB mAb was abrogated in SCID mice as well as in mice depleted of CD4 and CD8.

**Fig. 5.** 4-1BB mAb treatment of mice with i.c. tumors leads to growth delay of concomitant s.c. tumors. Mice bearing either 3-day established i.c. or s.c. MCA 205, or both, were treated i.p. with 100 μg of 4-1BB mAb on days 3 and 6. A, survival of mice with either i.c. or i.c./s.c. tumors was significantly higher than untreated controls (P < 0.05). B, mice treated with 4-1BB mAb and bearing concomitant i.c. tumor had significant growth delay of s.c. tumors ( ). Arrows, mice that died of both i.c. and s.c. tumors. Bars, SE.
decreased CD4 cells (4%), whereas CD8 and CD4 cells in the untreated culture were 77 and 8%, respectively (19). In therapy experiments illustrated in Fig. 7, mice with 3-day established MCA 205 pulmonary tumors were treated with the adoptive transfer of $15 \times 10^6$ activated tumor-draining LN cells. In both cell doses, therapeutic effects of 4-1BB antibody-stimulated cells were significantly diminished as compared with cells generated in the absence of the antibody.

In previous studies, conjunctural treatment with systemic IL-2 enhanced the antitumor efficacy of T cell-mediated adoptive immunotherapy (20). We therefore tested whether the effector function of adoptively transferred tumor-draining LN cells could be enhanced by the concomitant administration of 4-1BB mAb. In experiments presented in Table 1, both 3- and 10-day established MCA 205 pulmonary tumors were treated with the systemic transfer of activated tumor-draining LN cells. Some groups of mice were also given 200 µg of 4-1BB mAb i.p. as a single dose on day 3 after cell infusion or as split doses of 100 µg on days 3 and 6. These doses of mAb alone showed very little antitumor effects against 3-day established pulmonary tumors (Fig. 2). Enumeration of pulmonary tumor nodules 17 days after tumor inoculation revealed that the conjunctural 4-1BB antibody treatment enhanced the therapeutic efficacy of the transferred cells. Because treatment of pulmonary tumors with 4-1BB mAb was ineffective, the observed enhancement was likely to be the result of transferred T-cell activation by the administered antibodies.

**DISCUSSION**

Interactions between the costimulatory molecule on antigen-presenting cells and its counterreceptor on T lymphocytes play a key role in the induction of cell-mediated immune responses (21). By transfection of B7.1 or B7.2 genes into murine tumor cells, protective and sometimes curative immunity against wild-type tumors has been induced in several model systems (5). Further analysis has indicated that the immunogenicity of tumors, as determined by immunization and challenge experiments, is critical to the effects of B7 costimulation on tumor immunity (6). The growth and rejection of B7-transfected immunogenic, but not nonimmunogenic, tumors support the view that both an antigen-specific signal 1 and nonspecific costimu-
Table 1 Enhancement of therapeutic effects of adoptive immunotherapy by concomitant α4-1BB administration

<table>
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<th>No. of cells transferred</th>
<th>α4-1BB treatment</th>
<th>Mean no. of pulmonary metastases (SE)</th>
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ND, not done.

* Significantly different from the group of mice receiving the same number of activated LN cells but not treated with 4-1BB mAb (P < 0.05).

The presence of an immune response to a tumor cell that was susceptible to the therapeutic effect of a mAb and the consequent development of antitumor immune responses suggests the involvement of a complicated series of interactions leading to enhancement of antitumor immune responses. By carefully examining the anatomical site of tumor, kinetics of T-cell activation, and the mode of immunotherapy, the use of 4-1BB mAb may prove to be beneficial for the treatment of human cancer.
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