[CANCER RESEARCH 61, 2031–2037, March 1, 2001]

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

Julian A. Kim,[2] Bruce J. Averbook, Kathleen Chambers, Kevin Rothchild, Jorgen Kjaergaard, Robert Papay, and Suyu Shu

Department of General Surgery and Center for Surgery Research, Division of Surgery, Cleveland Clinic Foundation, Cleveland, Ohio 44195 [J. A. K., K. C., K. R., J. K., S. S.], and Department of Surgery, Metro Health Medical Center, Cleveland, Ohio 44109 [B. J. A.]

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 (i.c.), 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 (LNs), administration of 4-1BB mAb had adverse effects, despite the apparent hypertrophy of the LNs. 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 include CD40, CD27, CD30, and OX-40. 4-1BB expression is generally seen ~48 h after stimulation of T cells with immobilized anti-CD3 and/or anti-CD28 antibodies, and expression is maintained for 4–6 days (1, 2). Engagement of 4-1BB by 4-1BBL[3] found on antigen-presenting cells may lead to generation of cytolytic T cells as well as promotion of T-helper 1 cells, which produce high levels of IFN-γ (3).

There is substantial evidence that 4-1BB may play a role in the development of antitumor immunity. mAbs have been developed that bind to 4-1BB and provide a costimulatory signal to activated T cells that is distinctly different from CD28-mediated costimulation. It has been demonstrated 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, cells generated demonstrated 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 (*Pkrdc*^{SCID}) 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 dimethylhydrazine-induced colon adenocarcinoma of B6 origin obtained from the laboratory of S. A. Rosenberg (NIH, Bethesda, MD; Ref. 11). The GL261 tumor, a 3-methylchlolanthrene-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% CO_2. Cells were harvested by brief treatment with 0.25% trypsin/0.02% EDTA (Life Technologies, Inc.) and resuspended in HBSS for animal inoculations.

Received 7/5/00; accepted 12/28/00.

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[1] Supported by USPHS Grants R01-CA78263 and R01-CA67324 from the National Cancer Institute.

[2] To whom requests for reprints should be addressed, at Department of General Surgery Desk A80, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195. Phone: (216) 445-3800; Fax: (216) 445-3805; E-mail: kimj@ccf.org.

[3] The abbreviations used are: 4-1BBL, 4-1BB ligand; mAb, monoclonal antibody; LN, lymph node; SCID, severe combined immunodeficient; CM, complete medium; i.c., intracranial; IL, interleukin; RIg, rat immunoglobulin.

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, 1×10^5 tumor cells in 10 μl of HBSS were delivered into the right parietal lobe using a shielded 27-gauge needle at a depth of 4 mm, as described previously (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 Fekate's solution.

s.c. tumors were established in the flank by inoculation of tumor cells in 50–100 μl 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 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 1.5×10^6 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 4×10^6 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 ± SE for each group of five mice.

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). RIg 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 $P < 0.05$.

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 μg i.p. on days 3 and 6 after s.c. inoculation did not demonstrate significant antitumor reactivity (Fig. 1). Increasing doses to 300 μg on day 6 of treatment also failed to show significant tumor suppression (Fig. 1*A*, *Expt. 3*). Thus, for the three murine tumors, s.c.

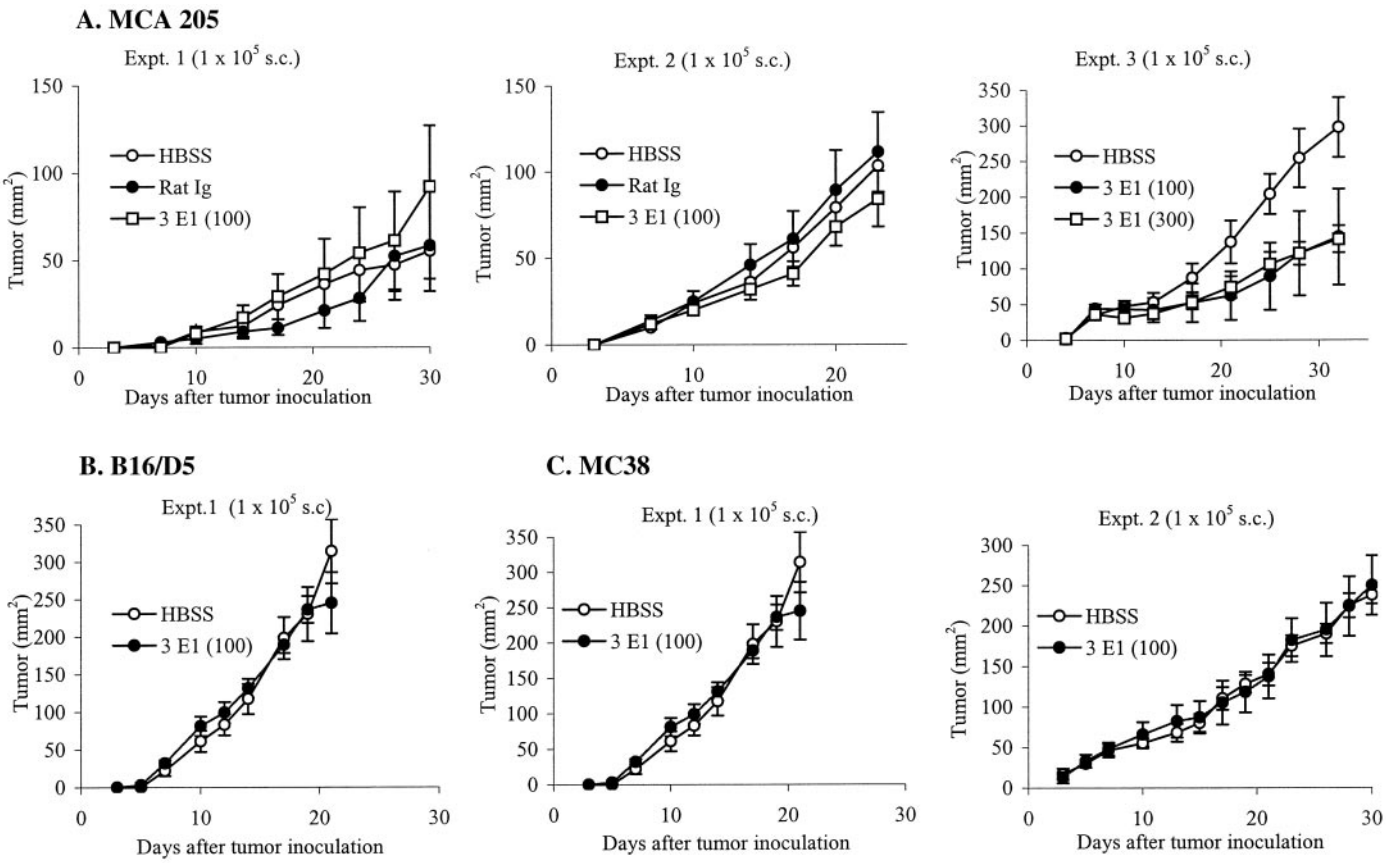


Fig. 1. Lack of significant therapeutic activity of 4-1BB mAbs against s.c. tumors. Mice bearing 3-day established s.c. tumors of MCA 205 (*A*), B16/D5 (*B*), or MC38 (*C*) were treated on days 3 and 6 with 4-1BB mAb 3E1, 100 μg/ml i.p. or 300 μg i.p. (*A*, *Expt. 3*). Data represent average tumor areas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

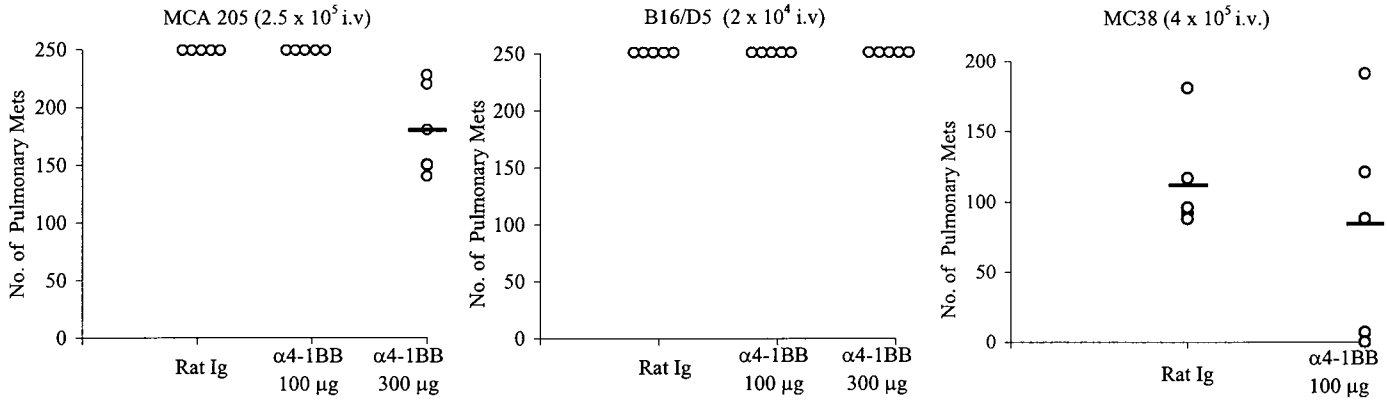


Fig. 2. Minimal therapeutic activity of 4-1BB mAbs against pulmonary tumors. Mice bearing 3-day established pulmonary tumors were treated with 4-1BB mAbs on days 3 and 6 at the doses indicated. Lungs were harvested 17 days after tumor inoculation, and metastases were enumerated. Mice with pulmonary tumors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

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 μg on days 3 and 6. Although no significant reduction of the number of pulmonary tumors was observed with 100 μg of mAb, increasing doses to 300 μg 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 RIg-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 μg 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. 4*A*). 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 1.5×10^6 MCA 205 tumor cells, none of the five cured mice were protected by lethal challenge (1×10^5) 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

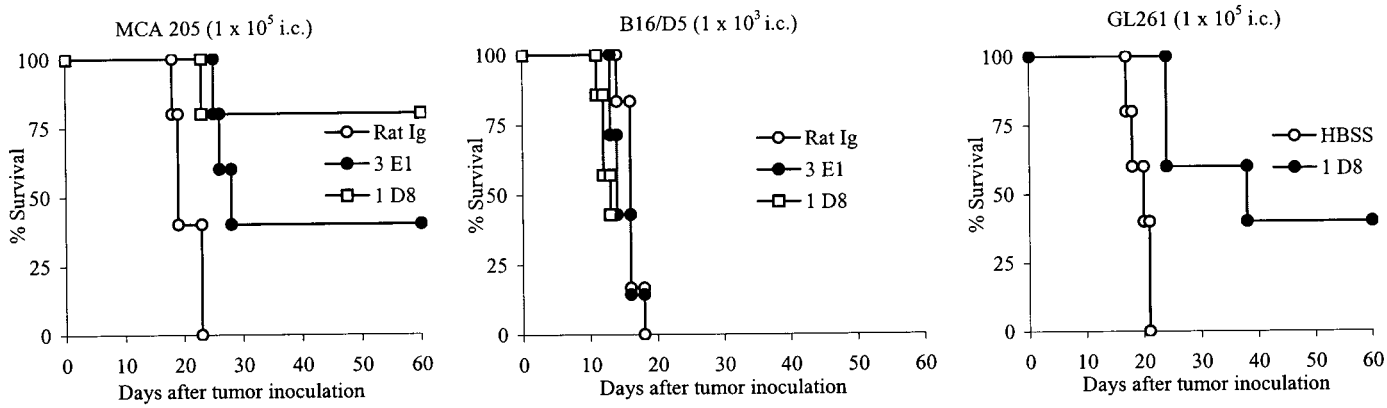


Fig. 3. 4-1BB mAb improves survival in mice bearing MCA 205 and GL261 but not B16/D5 i.c. tumors. Mice bearing 3-day established i.c. tumors were treated with either 4-1BB mAbs 3E1 or 1D8, 100 μg i.p. on days 3 and 6. Survival was significantly improved over control RIg-treated mice (*MCA 205*) or HBSS-treated mice (*GL261*) in mice treated with 4-1BB mAb ($P < 0.05$).

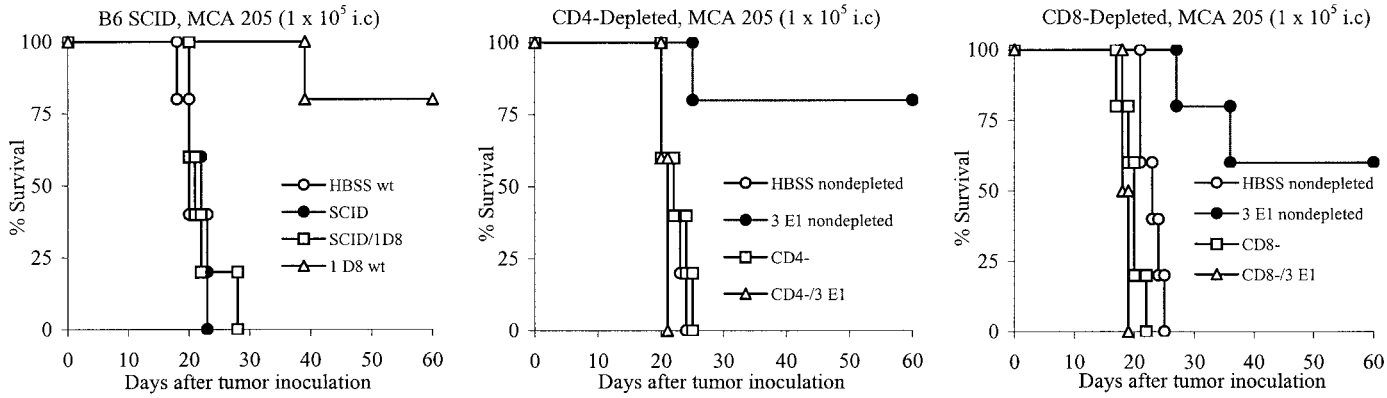


Fig. 4. Antitumor activity of 4-1BB mAb is dependent on both CD4 and CD8 T cells. Mice bearing 3-day established MCA 205 i.c. tumors were treated with 4-1BB mAb as described in Fig. 3. Survival advantage of groups treated with 4-1BB mAb was abrogated in SCID mice as well as in mice depleted of CD4 and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

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. 5*A*). 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. 5*B*). 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. RIg 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 ($22 \pm 6 \times 10^6$/LN *versus* $11.6 \pm 6 \times 10^6$/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

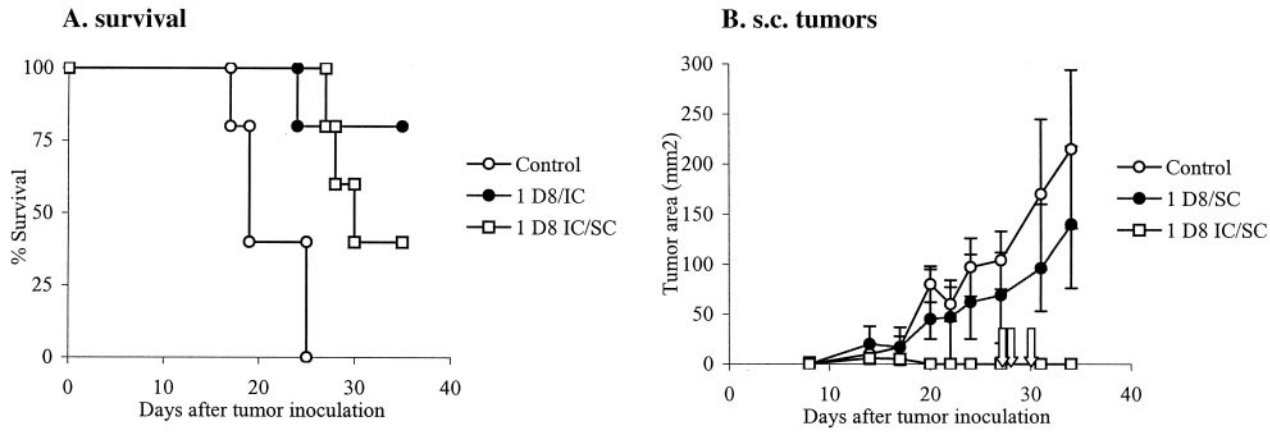
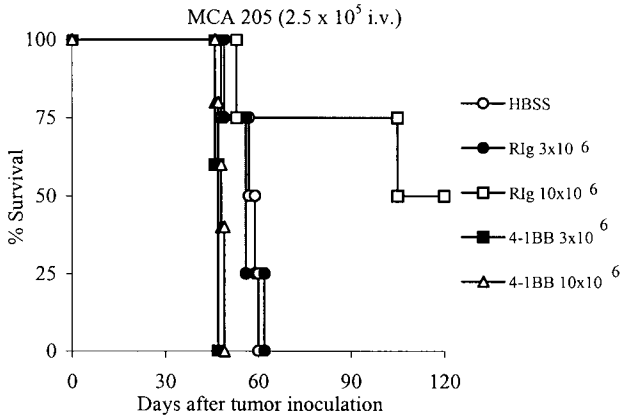


Fig. 5. 4-1BB mAb treatment of mice with i.c. tumors leads to growth delay of concomitant s.c. tumors. Mice bearing either 3-day established i.c. or s.c. MCA 205, or both, were treated i.p. with 100 μg of 4-1BB mAb on days 3 and 6. *A*, survival of mice with either i.c. or i.c./s.c. tumors was significantly 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.



Treatment:	*% positive cells*			
	Control Ig		4-1BB mAb	
	CD4	*CD8*	*CD4*	*CD8*
Fresh LN cells	24	22	23	30
Activated cells	15	60	8	67

Fig. 6. 4-1BB mAb inhibits sensitization of T cells in MCA 205 tumor-draining LNs. 4-1BB mAb was administered on days 3 and 6 after MCA 205 tumor inoculations. On day 12, tumor-draining LNs were excised and expanded with IL-2/anti-CD3 as described in "Materials and Methods." 4-1BB mAb-treated mice exhibited slightly higher percentages of CD8 cells and lower percentages of CD4 cells both before and after culture (below). However, therapeutic efficacy of activated tumor-draining LN cells from 4-1BB mAb-treated mice was diminished against 3-day established MCA 205 pulmonary metastases as compared with LNs from mice treated with RIg control.

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 15×10^6 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, conjunctional 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 conjunctional 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-

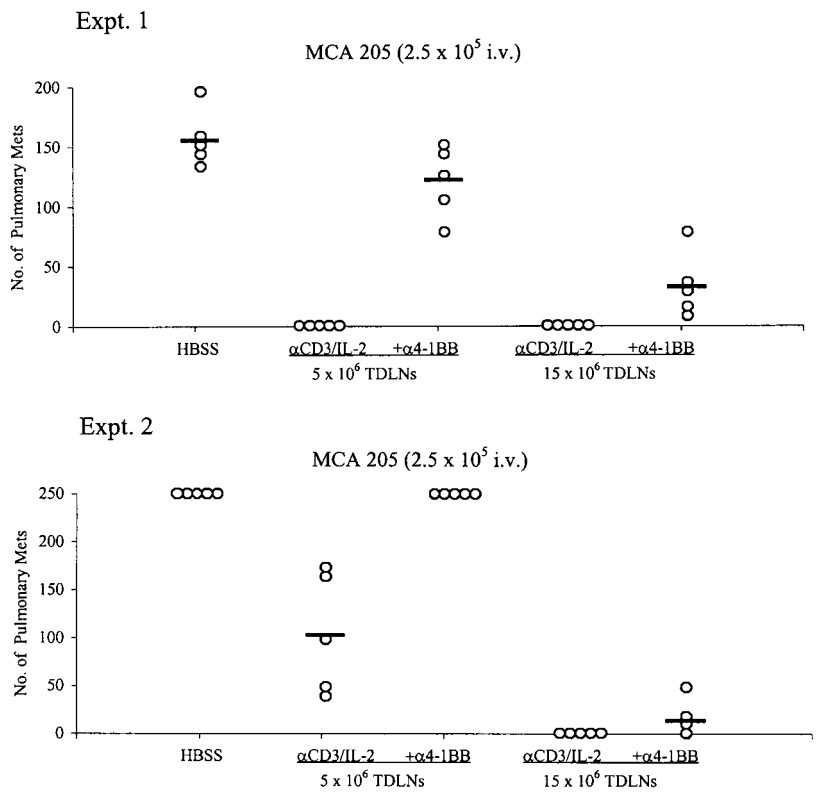


Fig. 7. 4-1BB mAb treatment of tumor-draining LN cells during culture suppresses the development of therapeutically effective cells. Day 12 MCA 205 tumor-draining LN cells were excised and cultured by the anti-CD3/IL-2 method in the presence or absence of 4-1BB mAb (10 μg/ml) for 2 days prior to expansion in IL-2. Activated LN cells were used to treat 3-day established MCA 205 pulmonary metastases. Mice treated with anti-CD3/IL-2 activated LN cells had significantly fewer pulmonary metastases as compared with mice treated with cells cultured in the presence of 4-1BB ($P < 0.05$).

Table 1 *Enhancement of therapeutic effects of adoptive immunotherapy by concomitant α4-1BB administration*

No. of cells transferred	α4-1BB treatment	Mean no. of pulmonary metastases (SE)	
		Day 3 tumors	Day 10 tumors
0	−	ND[a]	88 (18)
0	+	232 (36)	80 (26)
5×10^6	−	199 (38)	ND
5×10^6	+	32 (15)[b]	3 (2)
10×10^6	−	49 (24)	20 (12)
10×10^6	+	4.4 (2.1)[b]	1.2 (1.6)[b]
20×10^6	−	4.4 (4)	1.2 (1.9)
20×10^6	+	0	0.8 (1.2)

[a] ND, not done.
[b] Significantly different from the group of mice receiving the same numbers of activated LN cells but not treated with 4-1BB mAb ($P < 0.05$).

latory signal 2 are required for the amplification of T-cell immunity against tumors.

Different from the B7 receptor CD28, which is constitutively expressed on 95% of CD4 and 50% of CD8 T cells (7), the 4-1BB receptor molecule is expressed after T-cell activation through the T-cell receptor in the presence of cytokines or in combination with CD28 ligation (22, 23). Such a differential pattern of expression suggests a prominent role for B7/CD28 costimulation in priming naïve T cells and a role for 4-1BB/4-1BBL in the amplification and maintenance of an on-going, T cell-mediated immune response. The validity of this hypothesis has been supported by the observation that coexpression of 4-1BBL and B7.1 in the poorly immunogenic AG104A sarcoma enhanced the induction of effector CTLs and the rejection of wild-type tumor, whereas neither 4-1BB nor B7.1 single transfectants were effective (24). However, in a previous study (4), the administration of 4-1BB mAbs alone resulted in eradication of the same established large tumors in mice. The failure of gene-transfected tumor cells and the success of *in vivo* antibody administration to induce a therapeutic antitumor immune response can be explained simply by the extent of 4-1BB cross-linking mediated by the two approaches. These results also suggest that the "nonimmunogenic" AG104A tumor used is likely immunogenic, albeit weakly.

The results presented in the current study confirmed the antitumor effects of 4-1BB mAb for the treatment of immunogenic tumors, and these effects were T cell mediated with resultant systemic immunity and immunological memory. The failure of treating the poorly immunogenic B16/D5 tumor further supports the hypothesis that a minimum threshold of antigen-specific signal must be provided for the weak immune response to be amplified.

In our previous studies of adoptive immunotherapy, we have consistently found that the histological or anatomical locations of the progressive tumors had profound influence on the outcomes of treatment. For example, the treatment of 3-day established experimental brain metastases and s.c. tumors often requires sublethal irradiation, whereas there is no such requirement for the treatment of pulmonary metastases (25). Also notable is the enhancement of the therapeutic efficacy of T-cell immunotherapy of pulmonary tumors by the conjunctional administration of IL-2 (26). In contrast, similar IL-2 administrations after adoptive immunotherapy of i.c. tumors proved to be inhibitory to the therapeutic effect of the transferred T cells (13). Consistent with these findings, we observed that although i.c. tumors were susceptible to the 4-1BB mAb therapy, tumors grown s.c. or in the lung were refractory or resistant to the antibody treatment. It is intriguing that i.c. tumors were sensitive to the systemic antibody treatment, because the brain is considered to be an immunologically privileged site because of the existence of the blood-brain barrier and the lack of a lymph draining system. However, it has been demonstrated that the central nervous system contains specialized antigen-presenting microglial cells that, upon the influence of IFN-γ and other cytokines, express up-regulated MHC class I and II molecule expression (27). Thus, the growing tumors might have created an environment conducive to the initiation of an immune response. To support this was the observation of an abundant infiltration of T cells into the tumor mass (28). These tumor-infiltrating cells expressed OX-40R, indicating their early activation status and sensitization by the progressive tumor. It is therefore possible that 4-1BB ligation by the antibody amplified and expanded the immune reactivity of the infiltrating T cells in the i.c. tumor, leading to the subsequent development of systemic immunity. The sequential immunological events occurring after the 4-1BB mAb administration have been validated by the experimental observation that a s.c. tumor would regress during the rejection of i.c. tumor mediated by the 4-1BB antibody, whereas s.c. tumors alone failed to respond to the therapeutic effects of the antibody.

Because 4-1BB mAbs are known to enhance T-cell proliferation in the presence of a suboptimal concentration of an anti-CD3 mAb *in vitro*, we investigated their effects on the immune T-cell generation during the *in vitro* activation of tumor-draining LN cells. Despite an increased proliferation and a general shift of CD8 T-cell compositions, cells generated in the presence of 4-1BB mAbs demonstrated a diminished antitumor reactivity when adoptively transferred to tumor-bearing mice. At first glance, 4-1BB antibodies either inhibited the proliferation of tumor-immune T cells or augmented the proliferation of T cells of irrelevant specificities *in vitro*. However, it is more likely that the paracrine effects of 4-1BB expression and IL-2 production by the responding T cells led to a heightened concentration of this T-cell growth factor in the culture medium, which alone may have contributed to the generation of less effective T cells, as documented previously (29). Similarly, the *in vivo* administration of 4-1BB mAb during LN sensitization stimulated hypertrophy of the nodes, but the resulting cells were not as effective as those from animals without the antibody treatment. However, the mechanism of the suppression is not clear currently.

In adoptive immunotherapy, the outcome of therapy is related not only to the number of effector T cells transferred but also to the length of time the transferred T-cells survive *in vivo*. Therefore, any conjunctional regimen of treatment that will promote *in vivo* expansion and survival of the donor T cells will likely improve therapeutic efficacy. Immune T cells activated *in vitro* can be induced to grow *in vivo* to much more substantial numbers by the administration of exogenous IL-2 (30). In this study, we found that the administration of 4-1BB mAb shortly after the T cell transfer augmented the therapeutic effects of adoptive immunotherapy. The conjunctional use of 4-1BB mAb for adoptive immunotherapy may have the same effects of exogenous IL-2 treatment but sparing the severe toxicity associated with the clinical use of IL-2. There is also the recent experimental evidence that in this setting anti-4-1BB mAbs may be providing a "survival signal" that enables T cells to survive and perform their effector function after the antigen-specific activation process (31). It is noteworthy that studies in allograft rejection suggest that 4-1BB ligation may up-regulate the proliferation of CTLs without additional IL-2 (32).

In summary, our experience with the *in vivo* 4-1BB ligation on activated T cells by 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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Cancer Res 2001;61:2031-2037.