ABSTRACT

We have shown previously that expression of the costimulatory ligand B7.1 by the UV-induced melanoma K1735 leads to rejection of the tumor by syngeneic hosts and the induction of immunity to challenge by the parental B7-negative tumor. Here we extend our analysis of the effectiveness of B7-positive tumor cells as vaccines to additional tumor models and analyze the protective immunity in detail. We have found that the immunity induced by K1735 is not restricted to the parental tumor cells but is effective against an additional melanoma line and an unrelated fibrosarcoma as well. This immunity is, however, relatively short-lived, and no significant protection is observed after 90 days. Depletion of CD4+ T cells prior to rechallenge has no significant effect on the subsequent rejection of B7-negative tumor cells. EL-4 thymoma cells transfected with B7.1 are also effectively rejected, and mice which have rejected B7 + EL-4 cells are immune to challenge with not only EL-4, but also reject an unrelated thymoma, C6V/L. In contrast to the short-lived immunity observed in the melanoma model, mice are effectively protected against challenge with EL-4 for longer than 90 days after rejection of B7 + EL-4. Finally, we show that irradiation severely diminishes the effectiveness of B7-positive tumor cells as immunogens. This work has implications for the use of B7-positive cells as tumor vaccines.

INTRODUCTION

The goal of antitumor immunotherapy is to induce a specific antitumor T-cell response which will be effective in eradicating disseminated tumor. Recent work has demonstrated that in addition to antigen-specific signals delivered through the T-cell receptor, costimulatory signals delivered through the T-cell surface molecule CD28 are necessary for full activation of T cells. Costimulation mediated through CD28 is necessary for the generation of IL-2-secreting CD4+ effector cells (1, 2) and for the generation of CD8+ cytotoxic T lymphocytes (3—5). B7 was shown to be the ligand for CD28 (6) and to provide costimulation to T cells when expressed by antigen presenting cell (APC) (7, 8). This molecule has since been designated CD80 or B7.1, and has been shown to be a member of the B7 gene family of costimulatory ligands, which also includes B7.2 (9—11).

In the absence of costimulation through CD28, CD8+ cells are largely dependent on help from CD4+ cells in the formation of IL-2 for the generation of CTLs; however, in the presence of B7-expressing APC, CD8+ T cells are freed from the requirement for help by CD28-mediated autocrine IL-2 production (5). In addition, it has been shown that the expression of B7.1 by target cells is not required for killing by CD8+ CTL (5).

Recently these observations have been applied to antitumor immunotherapy by inducing B7.1 expression by tumor cells in order to stimulate more effective antitumor immune responses (12, 13, 14). In each of these systems, B7-expressing tumors were rejected in vivo. We and others showed that a melanoma induced to express B7.1 is rejected in vivo and confers protection against subsequent challenge with non-B7-expressing melanoma (13, 12). In one system the additional expression of a xenogeneic antigen by the melanoma was required for both rejection and protection (12). Both groups demonstrated that CD8+ T cells were responsible for the rejection of the B7-expressing tumor. It has since been shown that the degree of effectiveness of rejection of B7-expressing tumors of various tissue origins correlates with the immunogenicity of the tumors, such that those tumors previously identified as immunogenic by standard immunization protocols are more likely to be rejected when induced to express B7 than are nonimmunogenic tumors (15).

In this study we show that the effectiveness of B7-mediated tumor rejection does not correlate with the expression of MHC class II molecules in a variety of tumor models. We present an analysis of the kinetics and specificity of, and the cellular basis for, the protective effect of exposure to a B7-transfected melanoma and a B7-transfected thymoma. We demonstrate the cure of an established thymoma using B7-transfected tumor cells and discuss the implications of these results for potential antitumor immunotherapies.

MATERIALS AND METHODS

Mice and Cell Lines

C3H/HeN and C3H/HeN-mu/mu female and castrated male mice were obtained from Fredrick Cancer Research Facility of the National Cancer Institute, Fredrick, MD. C57BL/6, DBA/2, and BALB/c female mice were obtained from Charles River Laboratories (Wilmington, MA). All mice were maintained according to NIH-approved procedures in the mouse colony at University of California at Berkeley and were used between 6 and 12 weeks of age.

The K1735 and CM519 melanoma lines, the CM5153 squamous cell carcinoma line, and the UV-5498-4 fibrosarcoma lines, all derived from UV irradiation of C3H/HeN mice (16), were gifts of Margaret Kripke (M. D. Anderson Cancer Center, Houston, TX). The BALB/c-derived, N-methyl-N-nitrosourea-induced SM1 mammary carcinoma line (17) was a gift of Dr. Satyabrata Nandi (University of California at Berkeley). The BALB/c-derived 51Blim1 colorectal carcinoma line was a gift of Dr. Robert Warren (University of California at San Francisco). The C57BL/6-derived thymomas EL-4 (18) and C6V/L (19) and melanoma B16 (20), as well as the DBA/2-derived mastocytoma P815, were also used in these studies.

The K1735, CM519, CM5153, UV5498-4, and 51Blim1 cell lines and their derivatives were maintained in Eagle’s MEM (University of California at San Francisco Cell Culture Facility, San Francisco, CA) supplemented with 10% fetal calf serum (Sigma Chemical Co., St. Louis, MO), nonessential amino acids, MEM vitamin solution, L-glutamine, sodium pyruvate, gentimycin (all from Irvine Scientific, Santa Ma, CA), and 7.5% sodium bicarbonate (Sigma). The EL-4, C6V/L, B16, and P815 cell lines and their derivatives were maintained in RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine, sodium pyruvate, gentimycin, and 2-mercaptoethanol. All cell lines were cultured at 37°C in 5% CO2 and were passaged weekly, using a 1—5-min incubation in 5 mm EDTA (Sigma) in HBSS (GIBCO, Grand Island, NY) or trypsin to remove adherent cells from culture dishes. Cells were carried in vitro for no more than 10 serial passages.

Transfection of Tumor Cell Lines

The cell lines listed in Table 1 were transfected with the SR1neo expression vector containing the B7.1 cDNA (kind gift of Lewis Lanier, DNAX Research...
Institute of Molecular and Cellular Biology, Palo Alto, CA) using a BTX T 800 electroporator (BTX, Inc., San Diego, CA) [5 pulses for 99 μs each at 450 or 600 V in 270 mm sucrose-7 mm NaPO4 (pH 7.4)-1 mm MgCl2]. Transfected cells were cultured for 24 h in complete medium at 37°C before the addition of selection medium containing 1–2 mg/ml Geneticin (G418 sulfate, Gibco). After 14 days of culture in selection, drug-resistant cells were cloned by limiting dilution in selective media.

**Flow Cytometry**

B7.1 expression was evaluated by staining with the B7-binding CTLA4-immunoglobulin fusion protein (gift of Peter Lane), followed by FITC-conjugated goat anti-human immunoglobulin (CalTag, South San Francisco, CA) second step reagents. MHC class I and II expression was evaluated by flow cytometry after staining the cells with an anti-pan MHC class I (M1–42398), anti-pan MHC class II (N22), anti-E-4 (14.4.45), or anti-A5 (10.2.16), followed by FITC-conjugated goat anti-hamster immunoglobulin or goat anti-rat immunoglobulin (CalTag) second step reagents. Flow cytometry was performed using FACSIV or FACScan cytometers (Becton Dickinson; San Jose, CA).

**Maintenance of Tumors in Athymic nu/nu Mice**

Cell suspensions of K1735- or CM5153-derived lines (2–5 × 10⁶ cells) were injected i.p. in the back of C3H/He-nu-nu mice and were sufficient to raise tumors in 100% of mice within 2 weeks. Tumors raised in nude mice were harvested and minced with razor blades into ~1-mm³ fragments. Fragments were implanted into other mice using a 19-gauge trochar needle for implantation s.c. in the back. Tumors were maintained by serial transfer in nude mice or started in nude mice from suspensions of cells which had been passaged fewer than 10 times in vitro.

**Growth of Tumor Transfectants in Vivo**

K1735 and CM5153 Derivatives. Solid tumors were raised in BALB/c-nu/nu mice or C3H/He-nu-nu mice from cell suspensions of the cloned transfecants. Tumor fragments (2 x 1 x 1 mm³) were implanted s.c. in the backs of C3H/He mice. Biometric diameters of the tumors were measured every third or fourth day. Mice were euthanized as tumors became large or necrotic, and the final tumor size, to a maximum of 100 mm³, was included in data calculation for subsequent time points.

EL-4 and P815 Derivatives. Single cell suspensions of 3 × 10⁶ B7-EL-4 or parental EL-4 were injected i.p. of groups of female C57BL/6 mice. Single cell suspensions of 1 × 10⁷ B7-P815 or parental P815 were injected i.p. of groups of female DBA/2 mice. Tumors grew as ascites and tumorogenicity was evaluated using mortality as an end point.

51BLim10, SM1, and B16 Derivatives. Single cell suspensions of 5 × 10⁶ B7-51BLim10 cells or V-51BLim10 were injected s.c. in the backs of groups of 10 female BALB/c mice. Single cell suspensions of 5 × 10⁶ B7-51SM1 cells or V-SM1 were injected s.c. in the backs of groups of 10 male BALB/c mice. Single cell suspensions of 1 × 10⁶ B7-B16 or parental B16 were injected s.c. in the backs of groups of 5 female C57BL/6 mice. Tumors were measured and the data were analyzed as described above for K1735 derivatives.

**Depletion of T-Cell Subsets**

Antibodies for depletion experiments were partially purified from ascites fluid by saturated ammonium sulfate precipitation. The partially purified ascites fluid was dialyzed versus PBS lacking azide and filter sterilized prior to use in vivo. The concentration of antibody in each preparation was determined by flow cytometry, using staining titrations of the partially purified ascites fluid on thymocytes compared to the staining by purified standards of known concentration.

Groups of 8 or 10 mice were given injections on 3 successive days with ammonium sulfate-purified ascites preparations of anti-CD4 [a gift from Dr. Warren], anti-CD8 [53.672, 0.5 mg/injection, (22)], a combination of anti-CD4 and anti-CD8 (total of 0.7 mg/injection), or a nonreactive control antibody [2.43, 0.7 mg/injection, (23)] for C3H/HeN or F536, 0.7 mg/injection for C57BL/6]. Three days after the last injection, depletion of each subset was verified by flow cytometry analysis of peripheral blood lymphocytes. Fragments of B7-K1735 were implanted s.c. or B7-EL-4 was injected i.p., tumors were measured as described above, and antibody injections were continued every 4–7 days. In the experiment shown in Fig. 7, depletion of CD8⁺ cells was not complete after 3 serial injections of mAb, and two additional injections of 1 mg/mouse were given on days 1 and 2 after challenge in the groups of mice which received anti-CD8, the mix of anti-CD8 and anti-CD4, or the control antibody. These groups were monitored for depletion again on day 6 after challenge, and CD8⁺ depletion in peripheral blood lymphocytes was >95%.

**Rechallenge of B7-K1735- and B7-EL-4-primed Mice**

Fragments of B7-K1735 were implanted between the shoulder blades s.c. in C3H/HeN mice or sham treated with empty trochar needles. On various days after priming, groups of treated, sham-treated, or naive mice were lightly anesthetized with Metofane (Pitman-Moore, Mundelein, IL) and were given implantations of CM519 tumor fragments (2 x 1 x 1 mm³) or UV5498-4 cell suspensions (1 x 10⁶) s.c. on the right and left dorsal sides, respectively. Mice were tested for the presence of tumor by palpation and scored as either positive or negative.

Groups of C57BL/6 mice were given i.p. injections of B7-EL-4 cell suspensions (3 x 10⁶ cells/mouse). Treated or naive mice were later given i.p. injections of suspensions of either EL-4 or C6VL (3 x 10⁶ cells/mouse). Tumor incidence was measured as mortality.

**Cure of Established Tumors**

Groups of C57BL/6 mice were given i.p. injections of single cell suspensions of 1 x 10⁶ EL-4 cells. These mice were subsequently given i.p. injections of single cell suspensions of 1 x 10⁶ B7-EL-4 cells or sham injected with PBS on successive days as indicated. Tumor incidence was measured as mortality.

**RESULTS**

**MHC Class II Expression Does Not Correlate with Rejection of B7-transfected Tumors**

A variety of murine tumor cell lines were transfected with the vector carrying the B7.1 cDNA, as described previously (13). The B7-transfected tumors included a thymoma (EL-4, Ref. 18), a colorectal carcinoma (51BLim10; gift of Dr. Robert Warren), a fibrosarcoma (CM5153, Ref. 16), a mastocytoma (P815), a mammary carcinoma (SM1, gift of Dr. Satyabrata Nandi, Ref. 17), and a second melanoma (B16, Ref. 20). The B7 transfecants, together with control transfecants or parental lines, were assayed for B7 and MHC expression by flow cytometry and tested for rejection in vivo. As shown in Table 1, expression of MHC class II molecules does not correlate with rejection of B7-transfected tumors. Accordingly, direct

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Rejection of B7 transfecant</th>
<th>MHC class I⁺</th>
<th>MHC class II (IFN-γ inducibility)⁺</th>
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<tbody>
<tr>
<td>K1735</td>
<td>+</td>
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<tr>
<td>EL4</td>
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<td>51BLim10</td>
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<td>B16</td>
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* Rejection of B7-transfected: +, no palpable tumor in ≥80% of treated mice 60 days after tumor implantation, or no mortality in ≥80% of treated mice 60 days after tumor injection; −, <10% difference in incidence from non-B7-expressing controls, which achieved ≥80% incidence by either mortality or tumor growth in all cases.

* MHC expression: +, increase in fluorescence of ≥5-fold over the fluorescence of the cells stained with the secondary reagent alone; −, no increase in fluorescence over the fluorescence of the cells stained with the secondary reagent alone.

* Cultured tumor cell lines were incubated with 100 units/ml recombinant IFN-γ (Genzyme, Cambridge, MA) overnight at 37°C prior to staining and flow cytometry, except for SM1, which was shown to express MHC class II after retroviral transduction of the cDNA encoding IFN-γ (S. Townsend and J. P. Allison, unpublished observations).
stimulation of CD4+, MHC class II-restricted T cells, by tumors cells is unlikely to be necessary for effective rejection of B7-expressing tumors.

**Protection Induced by B7-K1735 Is Short-lived, Whereas Protection Induced by B7-EL-4 Is Long-lived.** Our previous work demonstrated the ability of B7-K1735 to protect against challenge with V-K1735. In these experiments, B7-K1735 fragments were implanted s.c. in C3H/HeN mice and then challenged s.c. with fragments of V-K1735. These results demonstrated that B7-K1735 and V-K1735 are immunologically cross-reactive, and suggested that the expression of B7 is the functionally relevant difference between the two tumors. These studies were extended to test the kinetics of the induction of protection by challenging with V-K1735 at several different times after exposure to B7-K1735.

As shown in Fig. 1B, exposure to B7-K1735 induced effective protection against challenge with V-K1735 when the time interval between priming and challenge was 25 days. Protection was also effective after 33 days (Fig. 2, discussed further below) or 16 days (data not shown). In contrast, if the time interval between priming and challenge was only 5 days (Fig. 1A), protection was much less effective. Similarly, if the interval between priming and challenge was 60 (data not shown) or 90 (Fig. 1C) days, protection was minimal.

In contrast to the decay of protection in the melanoma system, long-lived protection was observed in a thymoma tumor model. The EL-4 thymoma grows as an ascites tumor following i.p. injection. B7-transfected EL-4 grew aggressively in athymic nude mice (data not shown) and was rejected in wild-type C57BL/6 mice, as shown in Fig. 2. In antibody depletion experiments similar to those described for the K1735 system, depletion of CD4+ T cells had no effect on the rejection of B7-EL-4, whereas the depletion of both CD4+ and CD8+ T cells allowed the growth of B7-EL-4 (Fig. 3). In addition, *in vitro* experiments have shown direct killing of EL-4 and B7-EL-4 targets by CD8+ T cells, and not CD4+ T cells, from B7-EL-4-primed mice (data not shown). Together, these data suggest that CD8+ T cells function as cytotoxic effectors in the rejection of B7-EL-4. To test for the induction of protective immunity, C57BL/6 mice were inoculated with i.p. injections of B7-EL-4 and then challenged 92 days later with i.p. injections of the parental EL-4. In contrast to the decay of protection provide by B7-K1735, protection conferred by B7-EL-4 to challenge by the parental tumor EL-4 persists for at least 92 days (Fig. 4), the latest challenge time tested.

**Immunization with B7-transfected Tumors Can Induce Cross-Protection against Challenge with Different Tumors.** The specificity of the protective immune response induced by B7-K1735 was evaluated. Mice which had previously rejected B7-K1735 were subsequently challenged with fragments of the parental tumor, and contralaterally, with either a second UV-induced melanoma (CM519) or a UV-induced fibrosarcoma (UV5498-4). In naive mice, neither of these tumors is as strongly tumorigenic in terms of tumor incidence as is K1735. Nevertheless, protection was apparent against challenge with either of these tumors (Fig. 5). The protection against these challenges was shown to have similar efficacy and kinetics to the protection against K1735 (data not shown). Accordingly, the protection induced by exposure to B7-K1735 was not tumor specific but was cross-reactive against both a second melanoma and fibrosarcoma.

Cross-protection was also observed in the EL-4 tumor model. In similar experiments, mice exposed previously to B7-EL-4 were challenged i.p. with a second syngeneic thymoma, C6VLB. As shown in Fig. 6, 100% of these mice rejected C6VL, whereas most of the naive mice succumbed to the C6VL challenge tumor. In this thymoma model, as in the melanoma model, the protective immunity induced by exposure to the B7-expressing transfectant cross-reacted against a different tumor.
CD8+ T Cells Are Responsible for the Protection Induced by B7-K1735. We have shown previously that CD8+ T cells and not CD4+ T cells are required for the rejection of B7-K1735 (13). In order to examine the role of T-cell subsets in the protective immunity induced by exposure to B7-K1735, T-cell subsets were depleted in vivo using mAb just prior to challenge on day 35 with V-K1735, and depletion continued after challenge. As shown in Fig. 7, depletion of CD8+ cells, and not depletion of CD4+ cells, allowed aggressive growth of the challenge tumor. This result demonstrates that CD8+ T cells are required for the protective effect induced by B7-K1735 and suggests that CD4+ T cells are not required. The rejection of the challenge tumor was not complete in any of the experimental groups in this experiment, in contrast to previous experiments. The kinetics of tumor growth (data not shown) suggested that all of the mice, including those injected with control antibody, were immunocompromised by the numerous depleting injections.

Irradiation Abolishes the Protective Effect of B7-K1735. Therapeutic approaches utilizing B7-transfected tumors will require the use of inactivated cells or otherwise innocuous forms of immunogen. In order to test the potential usefulness of irradiated cells for such approaches, we tested whether irradiation of the immunizing tumor at a level sufficient to stop tumor cell growth would affect the ability of the fragments to induce protection against subsequent challenge with the control tumor. As shown in Fig. 8, irradiation of B7-K1735 tumor fragments prior to injection of the mice completely eliminated the ability of the cells to induce protection against challenge with V-K1735 or UV5498-4. This result suggests that irradiation may not be an appropriate method for inactivating tumor cells for use in vivo, and that alternative methods for inactivating cells may be required.

Cure of Established Tumors. In order to test the potential for curing established tumor using B7-transfected tumor cells, C57BL/6 mice were inoculated i.p. with parental EL-4 cells and then treated with serial i.p. injections of B7-EL-4 cells or PBS. As shown in Fig. 9, all of the injection regimens effectively cured 80–100% of the tumor-bearing mice. In contrast to the EL-4 system, a wide variety of immunization protocols were tested for effectiveness in curing established K1735 tumors in a pulmonary metastasis model, and none of these protocols achieved effective cure (data not shown). These results demonstrate the potential of costimulation-mediated therapeutic strategies for the treatment of established tumors; however, the effectiveness will have to be determined for various tumor types.

DISCUSSION

The Role of MHC Class II Expression in B7-mediated Responses. We present here a comparison of the antitumor immune responses induced by B7 expression by a variety of tumors, including tumors which express both MHC class I and class II, and tumors which express only MHC class I. It has been suggested that activation of CD4+ helper cells, which are MHC class II restricted, may be necessary for the induction of a potent antitumor immune response. Experiments in which MHC class II transfection of tumors induced rejection seem to support this hypothesis (24). In contrast, we show here no correlation between MHC class II expression by tumor and the rejection of B7 transfectants. One explanation for the previous results using transfection of MHC class II is that IL-2 provided by MHC II-restricted CD4+ T cells is likely to be required in responses against tumors which do not provide direct costimulation to CD8+ T cells. In contrast, the effectiveness of rejection of B7-expressing tumors which express only MHC class I supports the hypothesis that these tumors directly stimulate CD8+ T cells, eliminating the requirement for help by CD4+ T cells. Because MHC class I molecules generally present endogenously-derived peptides, a MHC class I-expressing tumor is the most likely candidate APC to prime a MHC class I-restricted response against itself.

The Kinetics of Protection. Detailed analysis of the induction of protection by B7 transfectants revealed differences in the longevity of protection. Protection induced by the B7-transfected melanoma was relatively ineffective when the challenge was given just 5 days after immunization with B7-K1735. The reactive T cells in these mice may not have expanded sufficiently in this short time to eliminate both the priming tumor and the challenge tumor. Consistent with this interpretation is the observation that coinjection of V-K1735 and B7-K1735 fragments at the same time results in the outgrowth of both tumors in some mice, rather than the rejection of one or both (data not shown). In this case the total tumor mass may overwhelm the capacity of the immune response to reject, the expansion of the tumors outgrowing the expansion of the immune response. In addition, the tumors may reach a critical mass which induces general immunosuppression before tumor-specific effectors lyse substantial numbers of tumor cells (25).

By day 60 after immunization with the B7-transfected melanoma, the capacity to protect against challenges wanes to about 50% (data not shown) and is minimal by day 90. Accordingly, the protection provided by prior exposure to B7-K1735 is effective only for a limited time.
Fig. 5. Treatment with B7-K1735 induces protection against challenge with a melanoma and a fibrosarcoma. (A) Groups of 5 mice which were either naive or B7-K1735 treated 25 days previously were challenged with s.c. implantations of CM519 tumor fragments. (B) Groups of 5 mice which were either sham treated or B7-K1735 treated 33 days previously were challenged with s.c. injection with a single cell suspension of UV5498-4.

Fig. 6. Treatment with B7-EL-4 induces protection against challenge with a second thymoma. Groups of 5 mice which were either naive or B7-EL-4-treated 92 days previously were given i.p. injections of a single cell suspension of C6VL.

period of time, from some point 5 days postinjection for at least 33 days. There are two possible explanations for this limited window of effectiveness. The first possibility is that exposure to B7-K1735 induces an effective primary immune response but generates no long-lived tumor-specific cells. In this case, once the primary response has been completely exhausted a secondary response could not be elicited. A second possibility is that long-lived cells are generated in the primary response to B7-K1735 but need costimulation to be amplified for an effective secondary response. The challenge tumor V-K1735 lacks B7, and thus would be unable to amplify these cells.

In contrast to the results with K1735 derivatives, protection induced by the B7-transfected thymoma is 100% effective for at least 92 days after immunization. The long-lasting protection conferred by B7-EL-4 suggests that costimulation on rechallenge is not required in all cases. This protection could be due to a more vigorous primary response that induced many more, or more effective effector cells, such that costimulation would be unnecessary to increase the precursor frequency on rechallenge. Alternatively, it is possible that some reservoir of EL-4 cells, but not K1735 cells, remain sequestered in vivo and chronically stimulate responder cells.

**Cross-Protection against Different Tumors.** The cross-protection by B7-K1735 against tumors of different tissue origin was surprising. Cross-reactive immunity between different UV-induced melanomas has been shown previously, but this cross-reactivity did not extend to UV-induced fibrosarcomas (16). Here we show that expression of B7 by a melanoma can induce cross-protection against a fibrosarcoma. In the melanoma experiments, mice were challenged either with fragments of V-K1735 or CM19 tumor raised in athymic nude mice, or with cell suspensions of UV5498-4. These separate techniques are unlikely to introduce similar artifactual exogenous antigens (i.e., bovine peptides in the case of the cell suspensions from tissue culture), and thus exogenous peptides are unlikely to account for the observed cross-protection in this tumor model. The cross-protection induced by B7-K1735, and by B7-EL-4, to other

Fig. 7. B7-K1735-induced protection against challenge tumor is abrogated by depletion of CD8+ cells and not CD4+ cells. Fragments of B7-K1735 were implanted in C3H/HeN mice on day 0; mice were then given injections of depleting mAbs starting on day 29. On day 35 after implantation, mice were challenged with fragments of V-K1735. Average tumor sizes on day 77 (day 22 after challenge) are presented for mice treated with anti-CD8 (10 mice), control mAb (5 mice), and anti-CD4 (5 mice). Columns, mean; bars, SD.
nontransfected tumors demonstrates that the vector used to transfect B7 does not provide relevant antigenic peptides.

It is possible that the melanomas and fibrosarcoma share tumor antigens that are recognized by tumor-specific T cells, although the tumors are derived from different tissues. The identity of transformation-associated tumor antigens would be of interest because such antigens could be useful for generalized antitumor vaccines, as well as provide insight into the mechanisms of transformation. The antigens could be derived from endogenous proviruses, such as murine leukemia virus, which are often activated on transformation (18). A variety of mutations in cellular genes has been shown to be involved in the process of transformation, and peptides derived from two of these mutant oncogenes have been shown to be immunogenic (26, 27, 28), although there is as yet no demonstration that oncogene-derived peptides are important in antitumor responses in vivo.

The antigens could also be normal cellular proteins, or embryonic proteins, which are shared between the tumors and are up-regulated in transformed cells (reviewed in Ref. 29). The antigens are unlikely to be tissue-specific antigens, as shown by the cross-reactivity between tumors of different tissue origins. In the case of the UV-induced melanoma, the antigen(s) is unlikely to be a result of the process of UV-induced transformation because subsequent in vitro cytotoxicity studies have shown that effectors derived from B7-K1735-treated mice also cross-react on an SV40-transformed renal carcinoma.

The cross-protection may instead point to a tumor-nonspecific component of the response induced by the B7-tranfectants, rather than to shared antigens. Such a response could be mediated by natural killer (reviewed in Ref. 30) or LAK (reviewed in Ref. 31) cells, both of which have been shown to have MHC-nonrestricted, antigen-independent activity. Murine natural killer cells express CD8 and have been shown to respond to stimulation through CD28 under certain conditions in vitro (32). LAK cells may be more likely to play a role because these cells express CD8, and we show here that CD8+ cells are responsible for the protection observed. LAK cells have been shown to require high levels of IL-2 for induction in vitro, and the requirements for induction in vivo are unclear. Another possibility is that a combination of tumor-specific and tumor-nonspecific activities are initiated in response to B7-transected tumors in vivo, and that tumor-specific cells may somehow regulate the activities of nonspecific cells. For example, CD8+ CTL can be isolated from the spleens of B7-EL-4-treated mice up to 120 days after immunization, whereas CD8+ CTL cannot be isolated from the spleens or draining lymph nodes of B7-K1735-treated mice. The relative contribution of different cell types may determine the vigor and longevity of the response. Further comparative analysis of the cellular basis for the short-term versus long-term protection in the K1735 and the EL-4 systems, respectively, will be necessary in clarifying these issues.

**Cellular Basis for Protection.** Antibody depletion in vivo demonstrated that CD8+ T cells are required for the protection conferred by B7-K1735, as well as for the initial rejection. This result raises the possibility of immunological memory mediated by CD8+ T cells, a subject of some controversy. The study of viral clearance in vivo in several laboratories has demonstrated that CD8+ effector cells can persist in the presence of antigen (33, 34), but also that true memory can be mediated by CD8+ T cells (35, 36). In our system, it is unclear whether the protection was mediated by residual effector cells or whether CD8+ memory cells were induced.

We and others have proposed that the key mechanism of B7-induced antitumor immune responses is the direct stimulation of tumor-specific CD8 T cells (13, 12). The work presented here supports this model in part, in that CD8+ T cells are shown to be responsible for protection against challenge tumor, as well as for the initial tumor rejection. The cross-protection data are more difficult to explain by this model, unless all of the cross-reacting tumors are shown to share the same relevant antigens. Another possibility is that the directly stimulated CD8+ effector cells may differentiate into tumor-nonspecific LAK cells, or that the stimulated CD8+ cells regulate the activity of another, tumor-nonspecific cell type.

**Implications for Antitumor Immunotherapies.** A successful antitumor immunotherapy will require the development of a potent immunogen that poses no risk to the recipient. A variety of approaches are being tested to inactivate B7-transfected tumor cells, while maintaining immunogenicity. The results presented here demonstrate that irradiated tumor fragments are much less effective than are freshly immunogen that poses no risk to the recipient. A variety of approaches are being tested to inactivate B7-transfected tumor cells, while maintaining immunogenicity. The results presented here demonstrate that irradiated tumor fragments are much less effective than are freshly irradiated tumor fragments used at the same dose. More intensive irradiation regimens (i.e., multiple boosts i.p.) are being tested to determine whether irradiated cells can induce protection, but the results raise the possibility that irradiated cells may not be useful...
therapeutically. In vivo, irradiated tumor fragments may quickly die and be scavanged, and thus not persist long enough to induce a potent immune response. Alternatively, irradiation may change the antigenicity of this particular tumor such that the relevant antigen is lost. The development of an effective innocuous immunogen will require a nicity of this particular tumor such that the relevant antigen is lost. Immune response. Alternatively, irradiation may change the antigenicity of this particular tumor such that the relevant antigen is lost.

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