Major Histocompatibility Complex Class II$^+$B7-1$^+$ Tumor Cells Are Potent Vaccines for Stimulating Tumor Rejection in Tumor-bearing Mice

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Summary

Mice carrying large established major histocompatibility complex (MHC) class I$^+$ sarcoma tumors can be successfully treated by immunization with genetically engineered sarcoma cells transfected with syngeneic MHC class II plus B7-1 genes. This approach is significantly more effective than previously described strategies using cytokine- or B7-transduced tumor cells which are only effective against smaller tumor loads, and which cannot mediate regression of longer-term established tumors. The most efficient tumor rejection occurs if both the class II and B7-1 molecules are coexpressed on the same tumor cell. Immunity induced by immunization with class II$^+$B7-1$^+$–transfected sarcoma cells involves CD4$^+$ and CD8$^+$ T cells, suggesting that the increased effectiveness of the transfectants is due to their ability to activate both of these T cell populations.

A number of different approaches have recently been developed to generate vaccines against tumors. Many of these approaches are based on genetically engineering tumor cells so they are more "immunogenic," and thereby stimulate the generation of tumor-specific effector cells. Some of these engineered tumor cells provide cytokines that bypass the need for helper cells (reviewed in reference 1), while others provide costimulatory signals for direct stimulation of CD8$^+$ T cells (2, 3) or CD4$^+$ T cells (4). In almost all cases, tumor cells that are genetically modified to secrete certain cytokines or to present costimulatory signals are themselves rejected by the autologous host, and are effective vaccines against subsequent challenge with wild-type tumor. Although such modified tumor cells are effective immunogens when administered before challenge with wild-type tumor, their effectiveness in mediating rejection of large or long-term preexisting tumors has not been established.

In the present report we describe an immunization strategy that is highly effective in mediating regression of established mouse sarcoma tumors. Our approach is designed to enhance, rather than bypass, the generation of tumor-specific T_h lymphocytes, and relies on the premise that activation of T cells requires the delivery of antigen-specific and costimulatory signals from the APC (5). Inasmuch as tumor peptides are the most likely targets for tumor-specific T cells, we have reasoned that MHC class I$^+$ tumor cells should be adequate targets for MHC-restricted effector lymphocytes, and that if provided with the appropriate restriction elements, they should also function as APC to class II–restricted T_h lymphocytes. We have therefore transfected class I$^+$ mouse SaI sarcoma cells with genes encoding syngeneic MHC class II plus B7-1 (previously called B7) costimulatory molecules and used the transfectants as immunogens for stimulating tumor-specific immunity.

Materials and Methods

Mice. Male and female A/J and nude mice of 6–10 wk of age were purchased from The Jackson Laboratory, Bar Harbor, ME, or bred in the University of Maryland Baltimore County animal facility.

Cells and Transfections. SaI, SaI/N, and all transfectants were maintained in culture as previously described (4, 6). Transfections were performed as previously described (6) using SaI/A^k clone 19.6.4 (6) or SaI. Cells were transfected with pSV2hph plasmid plus B7-1 cDNA (7). Bulk SaI/A^k/B7-1 transfectants were selected in 400 μg/ml hygromycin plus 400 μg/ml G418; SaI/B7-1 transfectants were selected in 400 μg/ml hygromycin. All lines were cloned at least once by limiting dilution. In some cases, B7-1 expression decreased with time in culture. These clones were recloned to obtain stable B7-1 expression. All transfectants used in this study are independent clones.

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Monoclonal Antibodies. 10-3-6 is a mouse IgG2a MHC class II I-A^k–specific mAb (8). 1G10 is a rat IgG2a B7-1–specific mAb (7). GK1.5 is a rat IgG2a $CD4^+$-specific rat mAb (9). 2.43 is a rat IgG $CD8^+$-specific rat mAb (10). 20-8-4 is a mouse IgG2a H-$2K^b$–specific mAb (11). Antibodies were protein A or protein G purified and used as previously described (4, 6).

Tumor Challenges. Ascities tumor challenges (SaI and derivatives) were performed as previously described (6). Mice were visually checked three times/wk for tumor growth, and when they became moribund they were killed. Tumor incidence is the number of mice with nonregressing tumors/total number of mice inoculated. In previous studies we have reported mean survival times rather than tumor incidence. Recent Institutional Animal Care and Use Committee (IACUC) regulations prohibit "survival to death" studies, hence we have changed end-points to measure tumor incidence. Solid tumor challenges (SaI/N) were given subcutaneously in the flank. Tumor diameters were measured three times/wk and the tumor volume determined by the formula πr^3. A tumor volume of 100 mm^3 is approximately equal to a tumor diameter of 6–7 mm. Mice reported as tumor-free did not have palpable tumors as long as they were maintained in our animal colony. The observation period for tumor incidence ranged from 53 d to 6 mo post-tumor challenge, depending on the experiment. In our experience, mice challenged with either the ascites or solid SaI line that were tumor-free 1 mo after challenge remained tumor-free indefinitely. Mice challenged with 10^5–10^6 wild-type SaI or SaI/N cells developed ascites or solid tumors within ~12–18 and 5–15 d, respectively. SaI-challenged mice became moribund with ascites tumor within ~20–28 d (6). Mice challenged with 10^5–10^6 SaI/N cells became moribund with large (>3 cm diameter) solid tumors by ~35–50 d after tumor challenge.

Immunofluorescence and Flow Cytometry. Immunofluorescence staining and flow cytometry analyses were performed as previously described using an Epics C flow cytometer (Coulter Corp., Epics Div., Hialeah, FL) (6).

In Vivo T Cell Depletions. Mice were depleted for $CD4^+$ T cells (GK1.5 mAb ascites) or $CD8^+$ T cells (2.43 mAb ascites) as previously described (4). Ascites of the mouse mAb 20-8-4 (11) was used as an irrelevant antibody control. Briefly, mice were inoculated with 100 μl of ascites fluid of the relevant mAb on days -6, -3, and -1 before tumor challenge (naive mice), or before treatment with SaI/A^k/B7-1 cells (mice carrying established tumor), and twice a week after tumor challenge until mice died of tumor, or until they became moribund, or until tumors became excessively large (usually 20–28 d after tumor challenge). Fluorescence analysis of splenocytes of treated mice demonstrated <3–5% or ≤1% of the normal level of $CD4^+$ or $CD8^+$ T cells, respectively.

Histopathology. Tumors were excised and fixed in 4% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Results

The mouse sarcoma I tumor is a methylcholanthrene-induced fibrosarcoma of A/J ($H\text{-}2^a$) strain mice. The SaI variant grows progressively as an ascites tumor when inoculated intraperitoneally, while the SaI/N variant grows as a solid tumor when inoculated subcutaneously. The wild-type tumor cells constitutively and stably express MHC class I molecules (K^k,D^d,L^d), and do not express MHC class II or costimulatory molecules. In previous studies we have demonstrated that SaI cells transfected with syngeneic MHC class II genes (SaI/A^k cells) are rejected by A/J mice and are a highly efficient vaccine against later challenge with wild-type class II^- tumor (6). Such transfectants must express the cytoplasmic domain of the class II heterodimer (12), or constitutively express the B7-1 costimulatory molecule (4). Because of the potent immunogenicity of SaI transfectants expressing truncated MHC class II molecules plus B7-1 molecules we have speculated that SaI sarcoma cells expressing full-length class II molecules plus B7-1 costimulatory molecules may be highly efficient immunogens in tumor-bearing mice (4). To test this hypothesis, we have generated transfectants that stably express both syngeneic MHC class II and B7-1 molecules, and compared them to tumor cells expressing B7-1 without MHC class II.

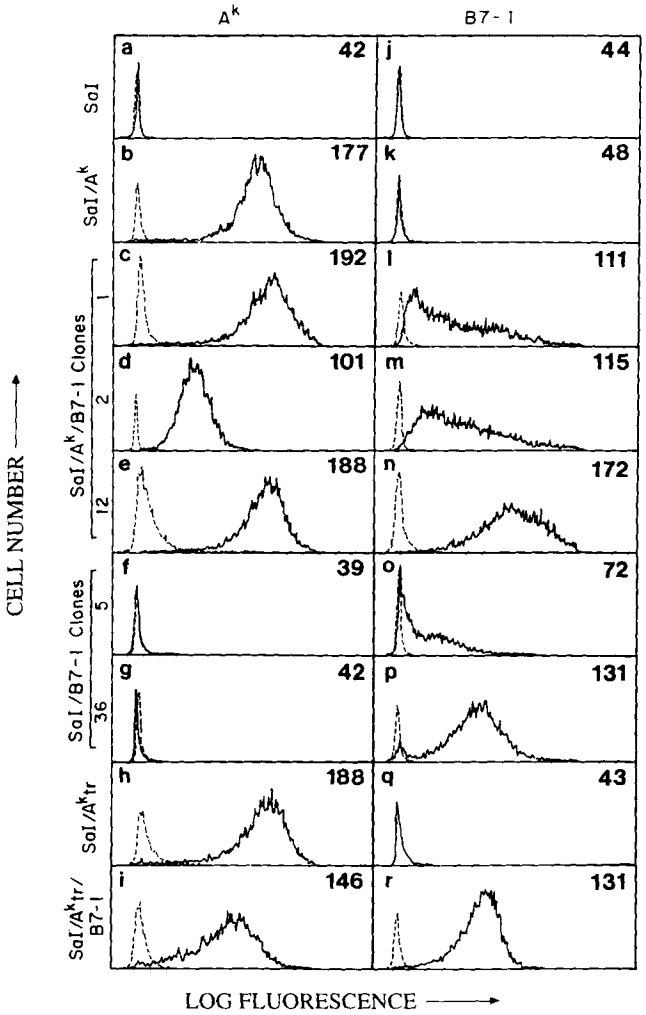

Figure 1. Immunofluorescence flow cytometry profiles of SaI sarcoma cells stained for MHC class II I-A^k and B7-1 molecules. Cells were stained by indirect immunofluorescence with mAb to I-A^k (10-3-6; 8) plus GAMIgG-FITC (*a–i*), or mAb to B7-1 (1G10; 7) plus GAratIgG-FITC (*j–r*). Dotted lines are staining of fluorescent conjugate without mAb; solid lines are staining with mAb plus fluorescent conjugate. Mean channel fluorescence for each clone is shown in the upper right-hand corner of each panel. The *x* axis represents three log cycles of fluorescence intensity.

Generation of SaI/B7-1 and SaI/A^k/B7-1 Sarcoma Cells. The ascites variants SaI and SaI/A^k cells were cotransfected

with the pRcCMV/B7-1 plasmid plus the pSV2hph plasmid to yield transfectants expressing B7-1 alone (SaI/B7-1 transfectants) or syngeneic MHC class II plus B7-1 molecules (SaI/A^k/B7-1 transfectants). Transfectants were screened by flow cytometry and cloned by limiting dilution one to three times. Fig. 1 shows flow cytometry profiles of wild-type SaI, class II^+ SaI/A^k clone 19.6.4, SaI/B7-1 clones 5 and 36, and SaI/A^k/B7-1 clones 1, 2, 12 stained for I-A^k and B7-1 molecules. Although the intensity of staining for B7-1 and I-A^k varies somewhat for the various transfectants, the mean channel fluorescence is between 72 and 172 for B7-1 and 101-192 for I-A^k. All of these lines have comparable levels of endogenously encoded MHC class I K^k and D^d molecules (data not shown). These transfectants have been maintained in vitro for several months, demonstrating their stable expression of MHC class II and B7-1 molecules.

Sarcoma Transfectants Expressing B7-1 Alone or B7-1 plus MHC Class II Are Rejected by Autologous A/J Mice. We have previously reported that SaI sarcoma cells expressing truncated MHC class II heterodimers plus B7-1 costimulatory molecules are rejected by autologous A/J mice (4). Simultaneously, other investigators have reported that K1735 melanoma cells transfected with B7-1 are rejected by autologous C3H mice (2, 3). To confirm these studies, we have challenged A/J recipients intraperitoneally with a range of tumorigenic doses of three independent SaI/A^k/B7-1 clones and 2 SaI/B7-1 clones, and followed them for tumor incidence. A/J mice have also been challenged four to five times at 7–10-d intervals with SaI/A^k/B7-1 clones 2 and 12, and SaI/B7-1 clone 36 to determine if exposure to multiple inoculations is tumorigenic. As shown in Table 1, none of the clones of either transfectant forms tumors in the autologous host at doses ranging from 10^5 to 6×10^6 tumor cells per mouse. These results confirm the observations that expression of B7-1 increases the immunogenicity of tumor cells and results in rejection of the transfectants.

Transfected Tumor Cells Expressing B7-1^+ or Class II^+ and B7-1^+ Molecules Induce Protective Immunity. The B7-1 molecule is required for the activation of T cells, but it is not required on the target cell for recognition by effector cells (13, 14). We have therefore inoculated autologous A/J mice with two different doses of SaI/A^k/B7-1 or SaI/B7-1 tumors, and 1–6 mo later challenged them with wild-type SaI ascites or SaI/N solid tumor cells. Tumor-challenged mice were observed for tumor incidence at least twice a week for 2–6 mo after tumor challenge. As shown in Table 2, three independent clones of SaI/A^k/B7-1 and two clones of SaI/B7-1 cells are potent immunogens against subsequent challenge of wild-type ascites (SaI) and/or solid (SaI/N) tumor,

Table 1. *Sarcoma Cells Expressing Transfected B7-1 and/or MHC Class II Genes Are Rejected by Autologous Mice*

Challenge tumor	Tumor dose	Tumor incidence*
SaI	10^5	5/5 (25 d)
	10^6	5/5 (21 d)
SaI/A^k/B7-1.1	10^6	0/5 (>53 d)
SaI/A^k/B7-1.2	10^6	0/5 (>6 mo)
SaI/A^k/B7-1.12	10^6	0/10 (>2 mo)
	5×10^6	0/2 (>2 mo)
SaI/A^k/B7-1.2 multiple injections	10^6	0/5 (>6 mo)
SaI/A^k/B7-1.12 multiple injections	10^6	0/5 (>6 mo)
SaI/B7-1.5	5×10^5	0/5 (>37 d)
SaI/B7-1.36	5×10^5	1/10 (>5 mo)
	10^6	1/15 (>2.5 mo)
	2×10^6	3/11 (>2.5 mo)
	6×10^6	0/2 (>3 mo)
SaI/B7-1.36 multiple injections	10^6	0/10 (>50 d)

Autologous A/J mice were challenged intraperitoneally with the indicated tumor cells and followed for tumor incidence as described in Materials and Methods. Multiply injected mice were given the indicated number of cells per injection at 7–10-d intervals for a total of four to five injections. Tumor incidence is the number of mice with tumors/total number of mice injected.

* Number in parentheses indicates how long after tumor challenge the mice were followed for tumor incidence.

Table 2. *Expression of Transfected MHC Class II and/or B7-1 Genes Confers Protective Immunity*

Immunizing tumor	Challenge tumor (dose)	Tumor incidence*
–	SaI (10^5 or 10^6)	10/10 (25 d)
–	SaI (5×10^6)	5/5 (23 d)
–	SaI/N (10^5)	9/10 (45 d)
–	SaI/N (10^6)	9/9 (42 d)
SaI/A^k/B7-1.1	SaI	2/5 (>6 mo)
SaI/A^k/B7-1.2	SaI	0/5 (>3.5 mo)
SaI/A^k/B7-1.12	SaI	0/15 (>5.5 mo)
SaI/A^k/B7-1.12	SaI/N (10^6)	0/5 (>8 mo)
SaI/B7-1.5	SaI	1/5 (>42 d)
SaI/B7-1.36	SaI	0/27 (>48 d)
SaI/B7-1.5	SaI/N (10^5)	0/4 (>42 d)
SaI/B7-1.36	SaI/N (10^5)	0/4 (>5 mo)

A/J mice were immunized intraperitoneally with 10^5–10^6 live tumor cells and challenged 1–6 mo later with SaI tumor (i.p.) or SaI/N tumor (s.c.). Challenge tumor doses are 5×10^6 cells unless noted otherwise. Tumor incidence is the number of mice with tumors/total number of mice injected.

* Number in parentheses indicates how long after tumor challenge the mice were followed for tumor incidence.

while unimmunized mice are highly susceptible. At the doses tested, class $II^+B7\text{-}1^+$ and $B7\text{-}1^+$ tumor cells are therefore equivalent in their ability to immunize naive mice against later challenges of unmodified tumor given as long as 6 mo after immunization.

SaI/A^k/B7-1 Cells, but not SaI/B7-1 Cells, Rescue Mice Carrying Established Tumor. Because of the potent protective immunity induced by the B7-1 transfectants, we have tested if the transfectants could be used as therapeutic reagents to treat A/J mice carrying established solid sarcoma tumor. This strategy is based on the assumption that mice carrying established tumors are not adequately stimulated by tumor peptide to mediate tumor rejection. If, however, the tumor-bearing host's immune system is optimally activated, then the resulting immune response may mediate partial or complete tumor regression.

To test the ability of the B7-1 transfectants to stimulate immunity in tumor-bearing mice, autologous A/J mice were inoculated subcutaneously in the flank with 10^5 SaI/N cells, and 13 d later given a single intraperitoneal inoculation of 10^6 SaI/A^k/B7-1 cells. Solid tumors were palpable at the injection site by days 5–7, and at the time of SaI/A^k/B7-1 administration SaI/N tumors were hard, solid, palpable masses of 2–5 mm in diameter. Histology of 10-d established tumors indicates that as early as 10 d after inoculation the malignant sarcoma tumors are highly invasive (Fig. 2 *A*), vascularized (Fig. 2, *A* and *B*), and contain areas of necrotic tissue, as well as mitotic tumor cells (Fig. 2 *C*).

As shown in Fig. 3 *A*, A/J mice not inoculated with SaI/A^k/B7-1 cells developed progressively growing SaI/N tumors that reached a diameter of ~2–3 cm and ulcerated by days 40–50. Of five A/J mice given a single injection of SaI/A^k/B7-1 clone 12 on day 13, however, the SaI/N tumors of three mice completely regressed, and the tumors of the two other mice were growth arrested (Fig. 3 *B*). In a similar experiment, three out of five mice showed regression of established SaI/N solid tumors after a single inoculation of SaI/A^k/B7-1 clone 2 on day 13 after initiation of SaI/N growth (data not shown). To determine the effect of the combination of B7-1 plus class II expression versus B7-1 expression alone, we have tested SaI/B7-1 cells in the same experimental protocol. As shown in Fig. 3 *C*, an equivalent dose of SaI/B7-1 cells has little, if any, effect on the growth of established SaI/N tumors. Likewise, inoculation of SaI/A^k cells not transfected with the B7-1 gene does not alter the growth of established SaI/N tumors (Fig. 3 *D*). Tumor cells transfected with MHC class II plus B7-1 genes are therefore efficient therapeutic agents for controlling wild-type tumor

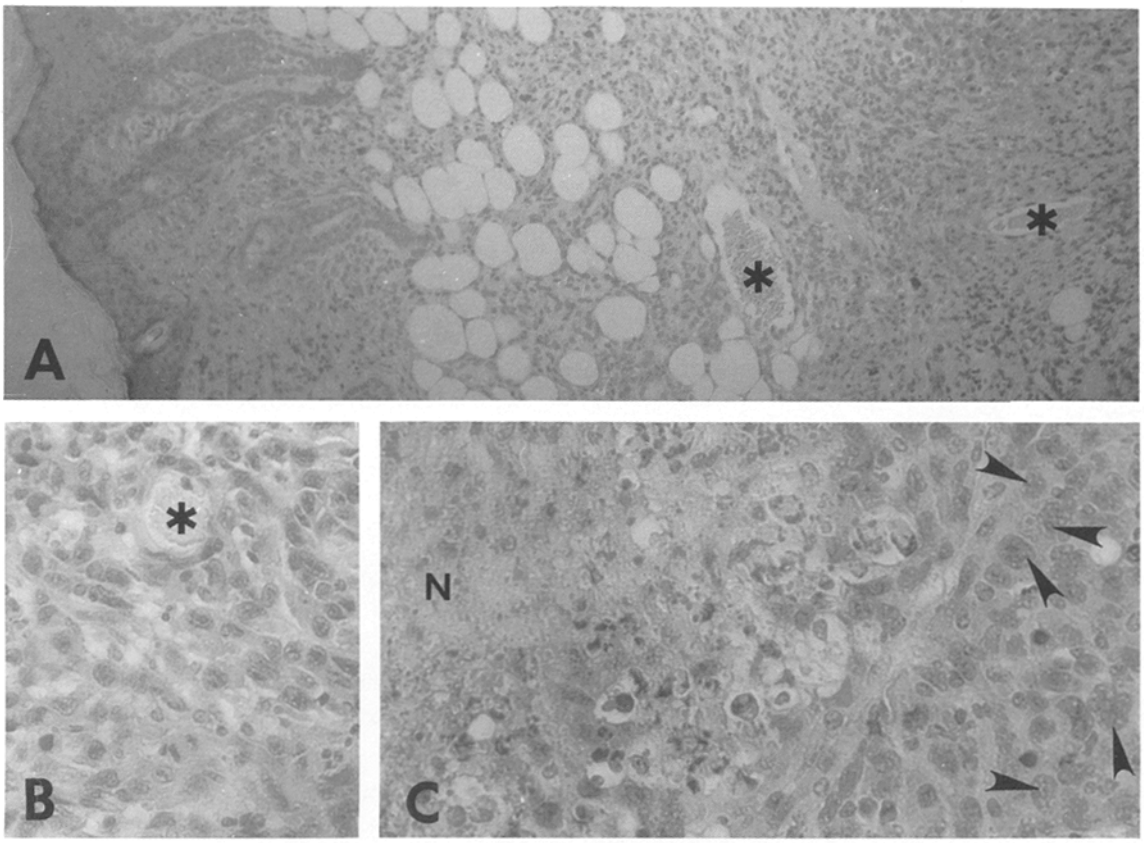

Figure 2. Histology of SaI/N tumors. Palpable tumors were excised from A/J mice 10 d after inoculation and prepared as described in Materials and Methods. *A* shows sarcoma tumor (*right* and *center*) interdigitating into normal skin epithelium (*left side of panel*) (×100). *B* shows a higher magnification (×250) of a blood vessel immeshed within the sarcoma tumor. *C* shows a necrotic region of the tumor (*left side*) and numerous mitotic tumor cells (*center* and *right*). * Denotes a blood vessel; → denotes mitotic cells; N denotes necrotic region.

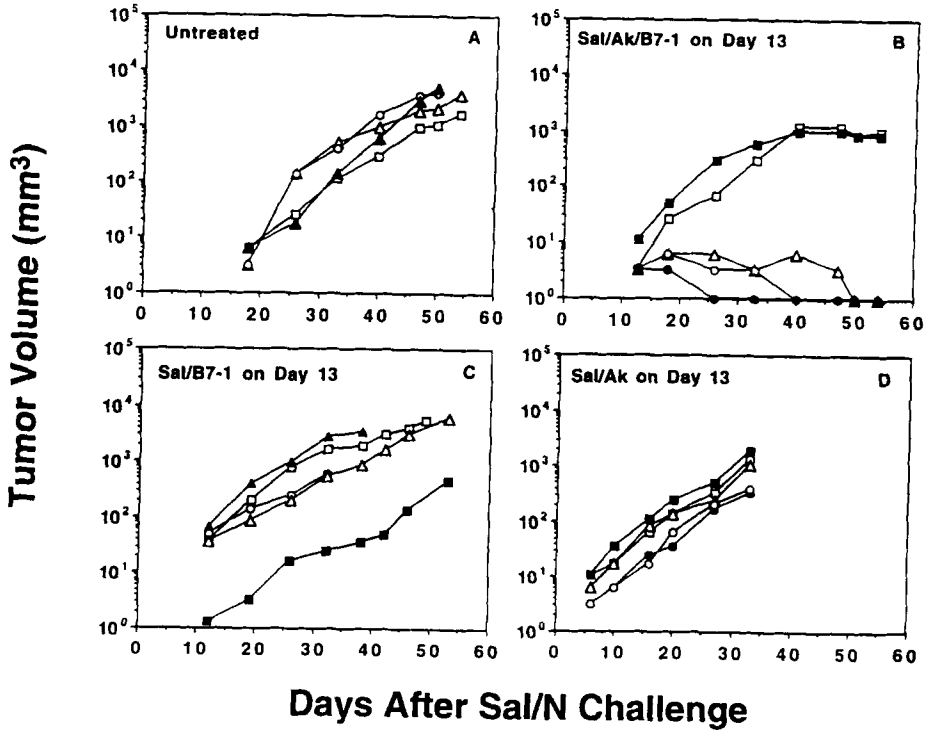

Figure 3. Inoculation of SaI/A^k/B7-1 cells into mice carrying 13-d established SaI tumors causes tumor regression. A/J mice were inoculated subcutaneously in the flank on day 0 with 10^5 wild-type SaI/N tumor and left untreated (*A*) or 13 d later given a single intraperitoneal injection of 2×10^6 SaI/A^k/B7-1.12 cells (*B*), 10^6 SaI/B7-1.36 cells (*C*), or 2×10^6 SaI/A^k cells (*D*). Each line represents growth of an individual solid tumor in an individual mouse. Cured mice remained tumor-free for the 2.5–7-mo observation period after SaI/N challenge. A second clone of SaI/A^k/B7-1 cells (clone 2) gave similar results as those shown in *B*.

growth in a significant percentage of animals carrying long-term established solid sarcomas.

Mice carrying larger solid tumors have also been treated with SaI/A^k/B7-1 cells. Autologous A/J hosts were inoculated subcutaneously in the flank with 10^6 SaI/N cells, and given SaI/A^k/B7-1 clone 12 cells intraperitoneally 9 d later. At the time of SaI/A^k/B7-1 inoculation, the mice had SaI/N tumors ranging in size between 4 and 7 mm in diameter. As shown in Fig. 4 *A*, untreated mice developed progressively growing tumors. Mice treated with SaI/A^k/B7-1 cells on day 9, however, showed a significant delay in SaI/N tumor growth, and two out of five of the mice showed complete tumor regression (Fig. 4 *B*). Similar delay in tumor growth and regression was seen when SaI/A^k/B7-1 cells were given 9 d after challenge with 3×10^5 SaI/N tumor (Fig. 4 *C* [untreated] vs. Fig. 4 *D* [given SaI/A^k/B7-1 clone 2 on day 9]) or 13 d after challenge with 10^6 SaI/N tumor (data not shown). SaI/B7-1 cells have also been tested for their ability to mediate tumor regression in animals inoculated with the higher SaI/N dose. A/J mice given 10^6 SaI/N cells on day 0, and treated with a single injection of SaI/B7-1 cells on day 9, do not have reduced tumor burdens relative to untreated mice (Fig. 4, *F* vs *E*). Larger SaI/N established tumors therefore also regress after SaI/A^k/B7-1 treatment, while inoculation of SaI/B7-1 cells has no significant effect on growth of established high or low dose SaI/N tumors.

Multiple Inoculations of Transfected Tumor Cells Are no More Effective than a Single Inoculation. We next investigated if multiple injections of SaI/B7-1 cells could cause regression of established SaI/N tumor. A/J mice were given 3.3×10^5 SaI/N cells s.c. on day 0 and subsequently inoculated intraperitoneally with SaI/B7-1 clone 36 cells on days 2, 11, 14, 23, 32, and 42. As shown in Fig. 5, no effect on SaI/N tumor growth is seen in mice given multiple inoculations of SaI/B7-1 cells, even if the first inoculation is given as early as day 2 after the initial tumor challenge (Fig. 5, *B* vs *A*).

We have also tested if the therapeutic potency of SaI/A^k/B7-1 transfectants is improved if multiple inoculations of transfected cells are given. A/J mice were given 10^6 SaI/N tumor cells s.c. in the flank on day 0, and inoculated 10, 15, 31, and 42 d later with SaI/A^k/B7-1 clone 2 cells. As shown in Fig. 5 *D* multiple dosing with SaI/A^k/B7-1 cells cures two out of five mice and significantly delays SaI/N tumor growth in the remaining three out of five mice (compare to Fig. 5 *C*). However the effect is not significantly better than that seen with a single inoculation of SaI/A^k/B7-1 cells (compare to Fig. 4 *B*).

SaI/B7-1 cells therefore have no effect on growth of established tumors even if administered in multiple doses, while SaI/A^k/B7-1 cells have an equal therapeutic effect if they are given as a single dose or as multiple inoculations.

Table 3 summarizes the therapeutic efficacy of the various SaI/A^k/B7-1 and SaI/B7-1 clones that have been tested for their ability to curtail the growth of established SaI/N sarcomas. Because the dose of therapeutic cells administered does not affect therapeutic efficacy, and because individual clones do not behave differently, we have pooled the results of independent clones and separate doses for this summary table. In all cases, mice "cured" by treatment with immunogenic SaI/A^k/B7-1 cells had tumors that completely regressed after treatment, and the mice remained tumor-free for the entire period of observation (2.5–7 mo after SaI/N challenge).

Rejection of SaI/B7-1 or SaI/A^k/B7-1 Tumor by Naive, Tumor-free Mice Requires CD4$^+$ T Lymphocytes. Previous ex-

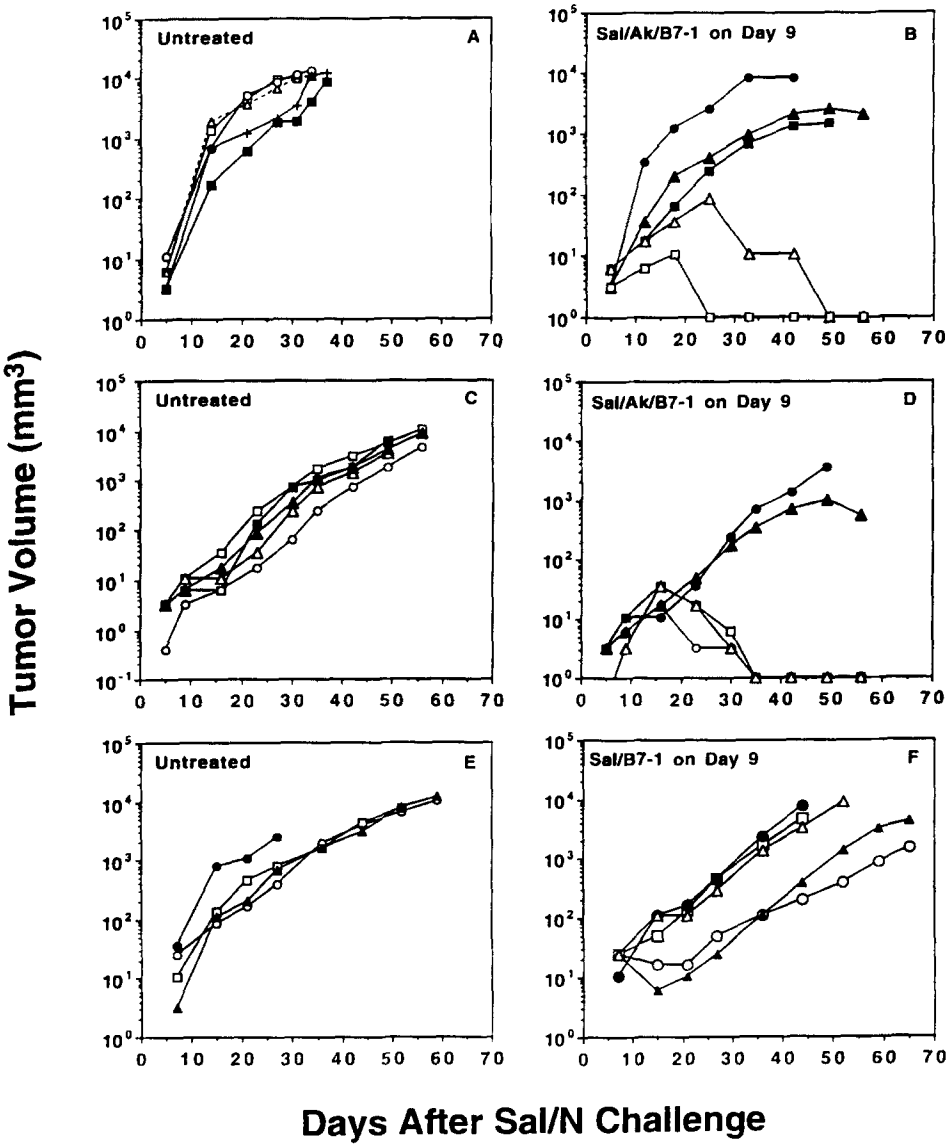

Figure 4. Inoculation of transfectants into mice carrying larger 9-d established tumors. A/J mice were inoculated subcutaneously in the flank on day 0 with 10^6 (*A*, *B*, *E*, and *F*) or 3×10^5 (*C* and *D*) wild-type Sal/N tumor cells, and left untreated (*A*, *C*, and *E*) or 9 d later given a single injection of 10^6 Sal/A^k/B7-1.12 cells (*B* and *D*), or 10^6 Sal/B7-1.36 cells (*F*). Parallel untreated control groups were run concurrently with each experimental group (untreated controls in *A*, *C*, and *E* for treatment groups in *B*, *D*, and *F*, respectively). Each line represents growth of an individual solid tumor in an individual mouse. Cured mice remained tumor-free for the 2.5–7-mo observation period after Sal/N challenge.

periments with class II^+ transfected Sal tumor cells have demonstrated that mice depleted of $CD4^+$ T cells are unable to reject tumor, suggesting that $CD4^+$ cells are required for tumor resistance. To determine if the immunity induced by B7-1 transfectants in tumor-free mice is also T cell–mediated, we have challenged sublethally irradiated (500 rads) autologous A/J mice with Sal/B7-1 or Sal/A^k/B7-1 tumor cells. In both cases, immunocompromised mice developed tumor (two out of two for each cell type), suggesting that immunity is T cell mediated. To confirm the role of $CD4^+$ T cells, A/J mice were in vivo depleted of $CD4^+$ T cells, and subsequently challenged with Sal/A^k/B7-1 tumor. As shown in Table 4, experiment 1, CD4-depleted mice develop tumor, while CD8-depleted mice do not. Development of immunity in naive tumor-free mice to the class II^+B7-1^+ tumor therefore requires $CD4^+$ T lymphocytes, but appears to be independent of $CD8^+$ T cells.

Immunotherapy of Mice Carrying Established Sal/N Tumors with Sal/A^k/B7-1 Cells Requires $CD8^+$ T Cells. In vivo depletion experiments have also been performed to identify the cells responsible for regression of established Sal/N tumors in Sal/A^k/B7-1–treated mice. Autologous A/J mice were inoculated subcutaneously with 10^6 Sal/N cells, and subsequently treated with 10^6 Sal/A^k/B7-1 cells on days 13–19 after Sal/N challenge. At the time of treatment with Sal/A^k/B7-1 cells, mice had solid tumors ranging from 4–8 mm in diameter. 9 d before receiving the therapeutic Sal/A^k/B7-1 cells, the mice were started on the regimen of mAb depletion. Administration of mAbs was continued for 3–4 wk or until animals became moribund or tumors regressed. As shown in Table 4, experiment 2, mice treated with the therapeutic Sal/A^k/B7-1 cells and either undepleted, CD4-depleted, or depleted with irrelevant mAb have a relatively low frequency of progressive tumor growth (38, 20, 20%

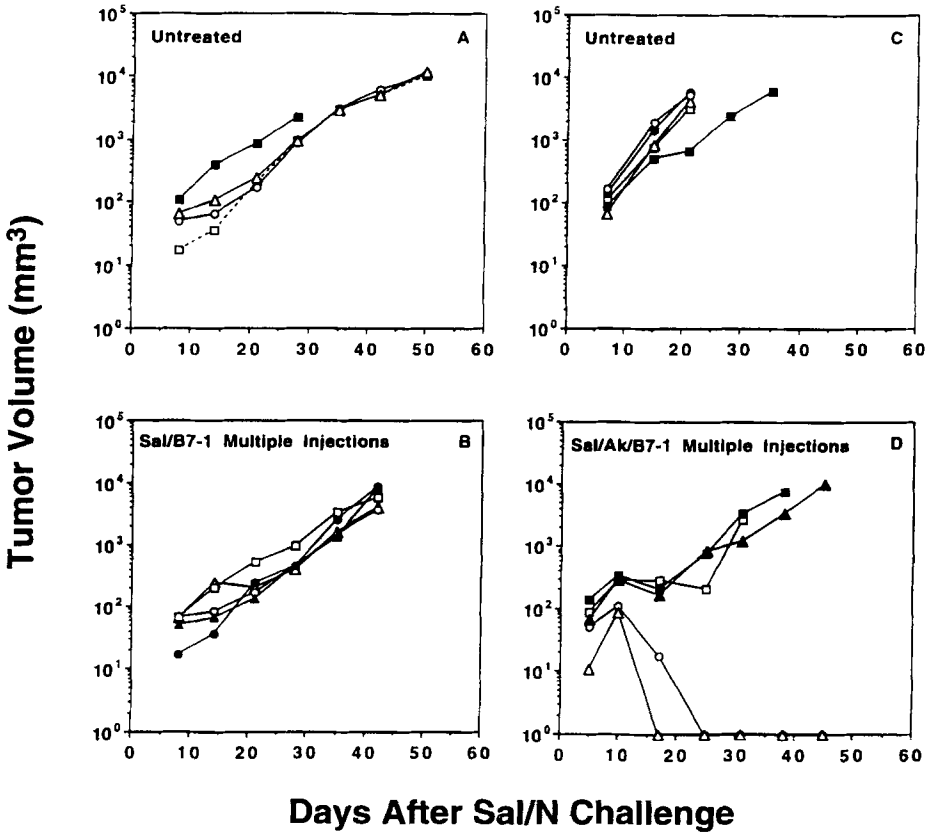

Figure 5. Multiple injections of SaI/A^k/B7-1 or SaI/B7-1 cells are no more effective than single injections in mediating tumor regression. A/J mice were inoculated subcutaneously in the flank on day 0 with 3.3×10^5 (*A* and *B*) or 10^6 (*C* and *D*) SaI/N cells and either untreated (*A* and *C*) or given $5 \times 10^5 - 10^6$ SaI/B7-1.36 cells on days 2, 11, 14, 23, 32, and 42 (*B*), or $4 \times 10^5 - 10^6$ SaI/A^k/B7-1.12 cells intraperitoneally on days 10, 15, 31, and 42 (*D*). Cured mice remained tumor-free for the 2.5–7-mo observation period after SaI/N challenge.

Table 3. *Summary Table of Mice Cured of Established SaI/N Tumors after Treatment with MHC Class II⁺B7-1⁺ Transfectants*

Initial dose of SaI/N tumor	Age of SaI/N tumor at time of treatment	Immunizing tumor	No. mice cured/total treated
10^5–3.3×10^5	–	–	0/11
	9–13 d	SaI/A^k/B7-1	9/15
	2–13 d	SaI/B7-1	0/10
10^6	–	–	0/14
	5–19	SaI/A^k/B7-1	19/33
	9	SaI/B7	0/10

Mice were inoculated subcutaneously in the flank with the indicated number of SaI/N cells. Tumors were allowed to become established in vivo, and tumor-bearing mice were then treated by intraperitoneal inoculation with the indicated immunizing cells. SaI/N tumor growth was followed for at least 5 wk, or until the tumors became >3 cm in diameter, at which time the mice were killed. A "cured" mouse is a mouse in which the SaI/N tumor disappeared, and the mouse remained tumor-free for at least 3 mo after treatment. Data are presented as pooled results of three (10^5 – 3.3×10^5 group) or four (10^6 group) separate experiments or experiments performed with different clones. In all cases, individual experimental groups showed the same partial responses seen in the pooled data.

tumor incidence respectively), while 100% of untreated, unimmunized, or CD8-depleted, immunized mice have progressively growing tumor. $CD8^+$ T cells are therefore required for successful immunotherapy of established tumor, while $CD4^+$ T cells are apparently not involved.

For Optimal Tumor Therapy, MHC Class II and B7-1 Molecules Must Be Expressed on the Same Tumor Cell. If coexpression of MHC class II plus B7-1 molecules is to be used therapeutically, it would be useful to know if the two molecules must be expressed on the same cell, or if separate cell populations, each expressing class II or B7-1, would be effective immunogens. Ideally we would test for complementation of B7-1 and class II by challenging mice with a mixture of two sarcoma clones, each expressing either B7-1 or class II, and neither of which was immunogenic by itself. Since sarcoma cells expressing truncated MHC class II molecules are malignant and unable to deliver a costimulatory signal (4, 12), they can be used as the population delivering the antigen-specific signal. SaI/B7-1 cells will deliver a costimulatory signal, however, they are themselves immunogenic (see Table 1). SaI/B7-1 cells are not, however, effective as rescuing cells (see Figs. 3, 4, and 5), and hence should not mediate rejection of SaI/A^ktr tumor cells unless there is complementation between the SaI/A^ktr and SaI/B7-1 cells.

Complementation was tested by challenging A/J mice intraperitoneally with a mixture of 5×10^5 or 10^6 SaI/A^ktr cells plus 5×10^5 or 10^6 SaI/B7-1 cells. As shown in Table 5, admixtures of SaI/A^ktr cells with SaI/B7-1 clone 36 cells

Table 4. *Immunity Induced by SaI/A^k/B7-1 Tumor Cells Requires CD4$^+$ T Lymphocytes in Naive Mice, and CD8$^+$ T Lymphocytes in Tumor-bearing Mice*

Experiment	Challenge tumor	mAb depletion	Immunizing tumor	Tumor incidence
1	SaI/A^k/B7-1	–	–	0/10
	SaI/A^k/B7-1	anti-CD4$^+$	–	5/5
	SaI/A^k/B7-1	anti-CD8+	–	0/5
2	SaI/N	–	–	9/9
	SaI/N	–	SaI/A^k/B7-1	5/13
	SaI/N	anti-CD4$^+$	SaI/A^k/B7-1	1/5
	SaI/N	anti-CD8$^+$	SaI/A^k/B7-1	5/5
	SaI/N	irrelevant IgG	SaI/A^k/B7-1	1/5

In experiment 1, autologous A/J mice were depleted for CD4+ or CD8+ T cells before challenge with SaI/A^k/B7-1 tumor. In experiment 2, SaI/N solid tumors were allowed to develop up to day 5 at which time A/J hosts were depleted for CD4+ or CD8+ T cells, or treated with control irrelevant ascites mAb. In all cases, mice were maintained on depleting mAbs for 3–4 wk. Tumor inocula for SaI/A^k/B7-1 cells were 10^6 cells i.p.; for SaI/N 10^6 cells s.c.

do not result in tumor rejection, while three clones of SaI/A^{k}tr/B7-1 cells (clones 1, 3, 6) are rejected. Control tumor challenges confirm that the SaI/A^{k}tr cells alone are malignant and that the SaI/B7-1 cells are rejected. If B7-1 and MHC class II are presented on the same cell, however, (SaI/A^{k}tr/B7-1 clone 1, 6, or 12) then rejection or partial rejection of the admixed SaI cells occurs. Optimal induction of tumor-specific immunity via coexpression of MHC class II plus B7-1 molecules therefore requires the presence of MHC class II and B7-1 molecules on the same tumor cell.

Table 5. *MHC Class II and B7-1 Molecules Must Be Expressed on the Same Tumor Cell to Stimulate Tumor-specific Immunity.*

Challenge tumor(s)	Tumor incidence*
SaI (5×10^5 or 10^6)	14/15 (25 d)
SaI/A^{k}tr (5×10^5 or 10^6)	15/15 (25 d)
SaI/A^{k}tr/B7-1.1	0/5 (>49 d)
SaI/A^{k}tr/B7-1.3 (10^6)	1/14 (>42 d)
SaI/A^{k}tr/B7-1.6	0/5 (>5.5 mo)
SaI/B7-1.36 (10^6)	0/5 (>6 mo)
SaI + SaI/A^k/B7-1.1	3/5 (>83 d)
SaI + SaI/A^k/B7-1.6	2/5 (>2 mo)
SaI + SaI/A^k/B7-1.12	0/5 (>2 mo)
SaI/A^{k}tr + SaI/B7-1.36	5/5 (25 d)
SaI/A^{k}tr (10^6) + SaI/B7-1.36 (10^6)	5/5 (25 d)

A/J mice were challenged with 5×10^5 cells i.p. unless noted otherwise. SaI/A^{k}tr cells are clone 6-11-8 (12). Some mice received a mixture of two tumor clones. Tumor incidence is the number of mice with tumor/total number of mice inoculated.

* Number in parentheses indicates how long after tumor challenge the mice were followed for tumor incidence.

Discussion

Numerous recent studies have demonstrated that tumor cells transfected or transduced with certain cytokine or B7-1 costimulatory genes become immunogenic (1–4, 15). Most of these studies, however, measure tumor immunogenicity by determining the tumorigenicity of the transfectants or transductants, or their ability to immunize non–tumor-bearing mice against later challenges with wild-type tumor. In one study, IL-12–secreting fibroblasts given concommitantly with wild-type tumor significantly delayed the onset of tumor growth (16). Similar results were seen when 4T07 mammary carcinoma cells were coinjected with IL-2–secreting 4T07 tumor (17). In only six studies have the transfectants/transductants been used under the more stringent situation of treating mice with established tumor. In two of these studies, B7-1$^+$ transfected melanoma cells coexpressing a strong viral antigen partially or completely blocked the development of melanoma metastases that had been induced 4–8 d before administration of the B7-1 transfectants (2, 18). In another study, injection of IL-4–secreting tumor cells into mice carrying small renal tumors (inoculation of 10^4 wild-type Renca cells given 6 d before IL-4 transfectants) resulted in tumor regression (19). In a third study, injection of B16 melanoma transduced with GM-CSF into mice carrying 3-d established B16 tumors (initial inoculum of 5×10^4 B16 cells) resulted in significantly improved survival. The GM-CSF–secreting B16 cells were significantly less effective against the higher tumor inoculum of 10^5 B16 cells (15). In another study, mice carrying established MBT2 bladder tumors, and given an inoculum of IL-2–secreting MBT2 cells on day 7 of primary tumor growth, showed significant reduction in tumor growth and extended survival (20). In the final study, treatment of 4-d established EL-4 tumors with B7-1–transfected EL-4 cells resulted in tumor regression in 60% of treated mice (21). It is difficult to quantitatively compare the effectiveness of these treatments because different tumors having different growth rates were used. However, these studies dealt with small tumors that had been

resident in the host mice for relatively short periods of time (≤8 days). In none of these studies using solid tumors were data presented demonstrating that the tumors were vascularized, and hence truly established. In contrast, MHC class II$^+$B7-1$^+$ tumor cells are highly effective in mediating regression of larger tumors that had proliferated in mice for up to 19 d. Although it is difficult to precisely compare the cytokine and class II,B7-1 therapy results because of the heterogeneity of the tumors used in the different studies, the class II$^+$B7-1$^+$ transfectants appear to be more effective immunogens for the treatment of larger and longer-term established tumor.

Recent studies by Chen et al. (21) indicate that the success of B7-1 in costimulating CD8$^+$ tumor-specific T cell responses is dependent on the inherent immunogenicity of the tumor. They suggest that coexpression of B7-1 by moderately or highly immunogenic tumors results in tumor rejection, while coexpression of B7-1 by poorly immunogenic tumors does not stimulate tumor rejection. In our experiments, the SaI sarcoma behaves as a moderately immunogenic tumor. Immunity can be induced by irradiated wild-type SaI, however, it requires at least 50-fold more irradiated wild-type cells (5×10^6) than class II$^+$ transfectants (10^5 or less). Furthermore, the immunity resulting from immunization with irradiated wild-type cells is only transient, while immunization with class II$^+$ transfectants yields 100% resistance for up to one year after immunization (22). Because the wild-type SaI tumor is not highly immunogenic, our experiments suggest that tumor cells with a wide range of inherent immunogenicity may be candidates for this therapeutic approach. Clearly, specific experiments with other moderately immunogenic, poorly immunogenic, and nonimmunogenic tumors are necessary to definitively demonstrate the scope of effectiveness of class II$^+$B7-1$^+$ transfectants as therapy for established tumors.

Expression of B7-1 without MHC class II converts the poorly immunogenic SaI sarcoma into an immunogenic tumor which is itself rejected by autologous A/J mice, and which is an effective vaccine against later challenge with wild-type tumor. We previously reported that an uncloned SaI/B7-1 line was malignant in A/J mice (4). Our current results with SaI/B7-1 clones appear to contradict these earlier findings. We now believe that the uncloned SaI/B7-1 line of our original report was malignant because many of the cells downregulated B7-1 expression in vivo, and hence the line became a mixture of SaI and SaI/B7-1 cells. Inoculation of the SaI/B7-1 line is therefore similar to a cell mixture experiment of SaI plus SaI/B7-1.36 in which five out of five inoculated mice die from tumor (Baskar, S., and S. Ostrand-Rosenberg, unpublished results). The SaI/B7-1 clones used in the present experiments were chosen for their stable expression of B7-1. Although SaI/B7-1 cells are immunogenic in naive mice, when used as a therapeutic agent to treat mice carrying established solid tumors, they have no effect, while SaI/A^k/B7-1 cells cause regression of wild-type established tumor. Rejection of an established tumor probably requires a more rigorous immune response than rejection of a freshly inoculated tumor. Likewise, immunization of a host against a later tumor challenge provides time to generate immunity in the absence of malignant tumor, and hence rejection of wild-type tumor by preimmunized mice is probably not as difficult as rejection of established tumors. SaI/B7-1 cells therefore induce immunity in non–tumor-bearing hosts, but are unable to induce sufficient immunity in tumor-bearing mice. In contrast, SaI/A^k/B7-1 cells appear to stimulate an immune response which is effective in mice carrying high tumor loads.

It is likely that the additional effectiveness of SaI/A^k/B7-1 vs SaI/B7-1 cells is because SaI/A^k/B7-1 cells can potentially stimulate CD4$^+$ and CD8$^+$ T cells, while SaI/B7-1 cells potentially stimulate only CD8$^+$ T cells. B7-1 is known to provide costimulation for activation of both CD4$^+$ and CD8$^+$ T cells (14, 23). Activation of CD4$^+$ T cells occurs if the B7-1 signal is delivered in conjunction with antigen presented by MHC class II, while activation of CD8$^+$ T cells occurs if the B7-1 signal is delivered in conjunction with antigen presented by MHC class I. Although the K1735 tumor cells used in the studies of Chen et al. (2) and Townsend and Allison (3) definitely express MHC class I molecules, their expression of MHC class II molecules is unclear. Chen et al. (2) do not specify if their K1735 variant expresses class II, while Townsend and Allison (3) state the tumor is class II$^+$, however, data supporting this assertion are not presented. Other investigators (24) as well as our own observations (Patterson, N., and S. Ostrand-Rosenberg, unpublished results) indicate K1735 cells do not express MHC class II molecules. These authors' findings that B7-1–transfected class I$^+$ K1735 cells induce CD8$^+$ T cell–dependent immunity is therefore consistent with the model that in the absence of MHC class II expression the K1735 tumor directly activates CD8$^+$ T cells and bypasses the generation of CD4$^+$ T cells. A more effective immune response may develop, however, if tumor-specific CD4$^+$ T cell help is also generated. Class II$^+$B7-1$^+$–transfected tumor cells that constitutively express class I may therefore stimulate a more potent immune response because they also activate CD4$^+$ tumor-specific T_h lymphocytes.

The CD4 and CD8 depletion data presented in this report (Table 3), support the hypothesis that the class II$^+$B7-1$^+$ tumor transfectants activate both CD4$^+$ and CD8$^+$ tumor-specific T cells. However, these data also raise additional questions about the mechanism by which the transfectants induce a therapeutic response. The depletion data for naive, non–tumor-bearing mice clearly demonstrate the involvement of CD4$^+$ T cells, while the depletion data for tumor-bearing mice demonstrate a critical role for CD8$^+$ T cells. Since wild-type class II$^-$ tumor cells are rejected by immunized, tumor-free mice, it is not clear why CD8$^+$ T cells are not needed for this response. Likewise, immunotherapy of established tumor appears to be independent of CD4$^+$ T cells, yet class II$^+$B7-1$^+$ tumor cells, and not tumor cells expressing B7-1 without class II, are able to mediate the effect. A trivial explanation for these results is that the mAb depletions were not complete, and that the small number of residual CD4$^+$ or CD8$^+$ T cells suffice for tumor rejection. Support for this explanation comes from two labs which recently demonstrated that $\beta 2$ microglobulin knockout, CD8-

deficient mice have <1% of the normal levels of CD8+ T cells, but are fully capable of rejecting class I allogeneic tumors (25, 26). Extremely low levels of CD8+ T cells therefore can be very effective in mediating tumor rejection. In constrast, in the present study the requirement for CD4+ T cells in tumor-free mice and CD8+ T cells in tumor-bearing mice, suggests that these particular lymphocytes are needed in greater than residual quantities. Regardless of the precise timing for involvement of CD4+ and CD8+ T cells in immunotherapy by class II+B7-1+ tumor cells, both of these lymphocyte populations appear to be required, supporting the hypothesis that the double transfectants are efficient inducers of tumor immunity because they successfully activate both CD4+ and CD8+ tumor-specific T cells.

Liu and Janeway (27) demonstrated in an in vitro system that maximal CD4+ T cell activation occurred when the antigen-specific signal and the costimulatory signal were delivered by the same APC. The mixed cell experiments of this report (Table 5) confirm the Liu and Janeway results, and extend their conclusions to in vivo generated immune responses. There are at least two explanations for why both signals must be expressed on the same APC. (*a*) The timing of delivery of the antigen-specific and costimulatory signals may be critical, and unless the two molecules are on the same APC, the timing requirement may not be met. (*b*) There may be steric considerations that prevent delivery of the two signals by different cells. Inasmuch as B7-1 levels are roughly equivalent on the various SaI/A^k/B7-1 and SaI/B7-1 transfectants it is unlikely that B7-1 density differences are responsible for the absence of sufficient T cell activation by SaI/B7-1 cells.

The finding that both the antigen-specific and costimulatory signals must be on the same tumor cell also has implications for tumor immunotherapy. If an immunization approach such as that described here is used clinically, the immunizing cells must be a cell population that homogeneously expresses both molecules. Strategies using third party cells such as fibroblasts to deliver the costimulatory signal would therefore not have a high likelihood of success.

Transfection of MHC class II and B7-1 genes into tumor cells was initially envisioned as enhancing tumor immunity by producing a tumor cell that could directly activate CD4+ T helper cells. Although class II molecules usually present exogenously synthesized peptides, there are numerous examples in which class II molecules present endogenously synthesized peptides (28–30). With the tumor cell as the primary APC, ample tumor peptide would be available for presentation by endogenously synthesized class II, and costimulation through B7-1 would be readily provided. The finding that both class II and B7-1 molecules must be expressed on the same tumor cell, although not definitive, is suggestive that the tumor cell is functioning directly in antigen presentation. Conversely, if the transfected tumor cells are not the principal APC, but third party (i.e., host) APC are involved, then the B7-1 costimulatory signal should be expressed on the host APC, and there should be no requirement for it on the tumor cells.

Although further experiments are required to ascertain the precise mechanism by which class II+B7-1+ transfectants stimulate immunity, these cells are clearly highly effective immunogens, and may have significant potential in future immunotherapy strategies for the treatment of established tumors.

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