Generation of Primary Tumor-specific Cytotoxic T Lymphocytes from Autologous and Human Lymphocyte Antigen Class I-matched Allogeneic Peripheral Blood Lymphocytes by B7 Gene-modified Melanoma Cells

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ABSTRACT

Expression of B7.1 costimulatory molecules on tumor cells has been shown to elicit antitumor immunity in mice. In the present study, we have developed a human B7.1 retroviral vector system to effectively transduce human melanoma cell lines and investigated the potential role of B7.1 in the generation of tumor-specific CTLs from peripheral blood lymphocytes (PBLs) in vitro. We have demonstrated that B7.1-modified melanoma cells are able to induce primary CTL activity from autologous, human lymphocyte antigen (HLA) class I-matched allogeneic PBLs and purified CD8+ T cells in the absence of exogenous cytokines. CTLs generated by B7.1 are tumor specific and HLA class I restricted, and CD8+ T cells are primarily responsible for this specific cytotoxicity. Furthermore, CTLs generated from HLA class I-matched PBLs by B7.1 are cytolytic to tumor cells autologous to the stimulated PBLs. These data suggest that B7.1-modified tumor cells can be used as a potent tumor vaccine for both autologous and HLA class I-matched allogeneic patients.

INTRODUCTION

Tumor-specific T cells recognize peptides derived from proteins synthesized by tumor cells and presented on their surface in the context of MHC gene products (1, 2). However, the progressive growth of tumors in the presence of an intact immune system suggests that the immune mechanisms have failed to defend the host from mutated cells or tumor cells (3). This failure is not due to the absence of recognizable tumor antigens (4) but is due to the inability of these antigens to effectively stimulate an antitumor immune response in vivo.

Studies have shown that full activation of T cells after antigen recognition is mediated by signals delivered by B7 and other costimulatory molecules (5–9). Among a large number of costimulatory molecules, including ICAM-1,3, leukocyte function-associated antigen 3, heat stable antigen (CD24), and CD40L, B7.1 plays an important role in antitumor immunity (8, 9). However, most tumors, particularly those of nonhematopoietic origin, do not express costimulatory molecules and thus would render T cells anergic or unresponsive for the specific antigens (7, 10). Studies in animal systems demonstrate that tumorigenicity is reduced significantly or abrogated following B7 transfection, and these gene-modified tumors were able to elicit specific antitumor responses (11–16). The antitumor immunity induced by B7 gene-transfected tumor cells is mediated by CD8+ (12, 14), CD4+ (15) T cell subsets, or both (16).

Recently, Harding and Allison (17) successfully generated primary tumor-specific CTLs in vitro using B7-transfected murine tumor P815 from naive allogeneic lymph node T cells, and Liu et al. (18) elicited tumor-specific CTLs in vitro from naive syngeneic spleen cells in the presence of low concentrations of IL-2 and IL-4, using three tumor cell lines (P815, EL4, and Lewis lung carcinoma) transfected with B7.1. However, in human systems, although proliferative activation by allogeneic (19, 20) and autologous (20) lymphocytes from PBLs costimulated by B7.1+ tumor cells have been reported, little is known about the specificity and HLA restriction of CTLs generated by B7.1-modified human tumor cells. In addition, there are, to our knowledge, no reports on in vitro generation of autologous CTLs from B7.1+ autologous tumor-stimulated lymphocytes.

In this study, we have developed a B7.1 retroviral vector system to effectively transduce human melanoma cell lines and investigated the capacity of B7.1 on tumor cells to reverse anergy in lymphocytes that have received antigen stimulation using both autologous and HLA class I-matched allogeneic PBLs or purified CD8+ T cells. We have shown that B7.1-modified melanoma cells are able to elicit tumor-specific, HLA class I-restricted CTLs in vitro from both autologous and HLA class I-matched allogeneic PBLs, and the CTLs generated by HLA class I-matched, B7.1-transfected melanoma cells are cytolytic to tumor cells autologous to the stimulated PBLs.

MATERIALS AND METHODS

Tumor Cell Lines. Fresh melanoma cells were obtained from surgically excised tumor-involved nodes, and assigned a sequential DM number, as described previously (21). The tumor cell lines were cultured in 75-cm2 flasks (Costar) with Eagle’s MEM (Life Technologies, Inc., Grand Island, NY) supplemented with 5% FBS (Life Technologies, Inc.), 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin. Cell lines were maintained as monolayer cultures and passaged by trypsinization with 0.0625% trypsin plus EDTA as required.

HLA Typing. Tumor cells or patient lymphocytes were HLA typed by a complement-mediated microcytotoxicity assay using HLA Comprehensive Phenotyping Trays (Gentra, Wayne, PA). Briefly, 1000–2000 lymphocytes or tumor cells were placed in each well of the typing trays and incubated for 30 min (for PBLs) or 60 min (for tumor cells) at room temperature. Following one wash, complement was added and the plates were incubated for an additional 30 min (for PBLs) or 4 h (for tumor cells). The plates were washed again, trypan blue was added, and the wells were scored microscopically for viability using the trypan blue exclusion test. Consistently, the HLA antigens expressed on tumor cells were expressed similarly on autologous lymphocytes. The HLA typing for the tumors and target cell lines used in this study is illustrated in Tables 1–3 and in the legends to Figs 1–6.

Isolation of Lymphocytes. Peripheral blood was collected in heparin from melanoma patients, diluted with HBSS, underlaid with lymphocyte separation medium (Litton Bionetics, Inc., Kensington, MD) and centrifuged at 1450 rpm for 40 min. The lymphocyte-rich layer at the interface was washed thoroughly with HBSS and counted. A portion of the lymphocytes was frozen in FBS plus 10% DMSO for later use.

Construction of Retroviral Vector Containing hB7.1 Gene and Tumor Cell Transfection. The full-length cDNA for human B7.1 was amplified by PCR from the plasmid pCDM8/hB7.1 (a gift from Dr. Freeman, Dana-Farber Cancer Institute, Boston, MA; Ref. 22). Primers were 5’-CTTAAAGAATTCTTATACAGGGCGTACACT (antisense) and 5’-GCTGCGGATCCCTCTAGGTATACAGGGCGTACACT (sense), which contain the EcoRI and BamHI

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3 The abbreviations used are: ICAM, intercellular adhesion molecule; IL, interleukin; DM, Duke melanoma; HLA, human lymphocyte antigen; FBS, fetal bovine serum; GM-CSF, granulocyte macrophage colony-stimulating factor; hB7.1, human B7.1; MTLR, mixed tumor lymphocyte reaction.
sites (underlined) for cloning. PCR amplification was performed under the following conditions: 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 45 s, followed by 72°C for 10 min. The PCR product was digested with EcoRI and BamHI, cloned into the EcoRI and BamHI-digested vector pLXSN (a gift from Dr. A. D. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA; Ref. 23), and introduced into competent bacterial HB101 by calcium precipitation (24). Vectors containing hB7.1 insert (named pLXSN/B7.1) were purified and transfected into the amphotropic packaging cell line, PA317 (23). Colonies were isolated by G418 (Life Technologies, Inc.) selection and expanded to cell lines, and cell-free supernatants were tested for the presence of virus. Cell lines expressing B7.1 (identified by flow cytometry) and secreting a high viral titer (about 10^9 colony-forming units/ml) were used to infect melanoma cells in the presence of 6 μg/ml Polybrene. The melanoma cells were selected with G418 (0.75—1.5 mg/ml, active concentration) for 2 weeks. G418-resistant cells were stained with FITC-conjugated anti-human CD80 (PharMingen, San Diego, CA), sorted on a FACStar Plus (Becton Dickinson, Mountain View, CA), and expanded to continuous cell lines.

**Immunofluorescence Phenotyping.** Tumor cells were incubated with optimal concentrations of FITC-conjugated monoclonal antibodies against HLA class I and II (Antigenix America, Franklin Square, NY), ICAM-1 (Immunotech, Marseille, France) or CD80 (PharMingen) for 45 min at 4°C. The cells were washed three times and resuspended in saline containing 1% formaldehyde. T cells were stained with FITC-conjugated monoclonal antibodies CD3, CD4, and CD8 (AMAC Inc., Westbrook, ME) as mentioned above. Analysis was performed on a Becton Dickinson FACScan flow cytometer (Becton Dickinson).

**Isolation of CD8^+ T Cells.** Nylon wool-enriched T cells from PBLS were further depleted of CD4^+ T cells by using M-450 CD4 Dynabeads (Dynal Inc., Oslo, Norway) as described in the manufacturer’s manual. Briefly, nylon wool-enriched T cells were incubated with M-450 CD4 Dynabeads (ratio of beads:T cells, 10:1) on ice with gentle rotation for 30 min. CD4-depleted cell suspensions were collected by subjecting the tube to a magnetic field for 2—3 min. The resultant cells contained >90% (92—96%) CD8^+ cells with no significant contamination (<1%) by CD4^+ cells (0.22—0.86%), as determined by flow cytometry.

**Induction of CTLS.** Lymphocytes (2—5 × 10^6/well) were cultured with irradiated (10,000 rads) tumor cells at a lymphocyte:tumor ratio of 20:1 in 2 ml of RPMI 1640 containing 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, and 5 × 10^-3 M 2-mercaptoethanol in 24-well tissue culture plates (Costar) at 37°C in a humidified atmosphere containing 5% CO2. The cells were fed on day 3 by removing the medium and replenishing the cells with an equal volume of fresh medium. The cells were harvested on day 5 for CTL assay.

**Human IFN-y Assay.** Target cells were labeled by incubation with 100 μCi of sodium ^51^Cr for 1 h at 37°C. They were then washed three times in HBSS and resuspended in RPMI 1640. The labeled target cells (5 × 10^5 cells/ml) were mixed with effector cells to yield several E:T ratios in 96-well U-bottomed plates and incubated at 37°C for 4 h. The supernatants were harvested and counted in a γ-spectrometer. The cytotoxic activity was calculated as follows:

\[
\text{Specific lysis} = \frac{(\text{Mean of experimental cpm} - \text{mean of spontaneous cpm})}{(\text{Mean of maximum cpm} - \text{mean of spontaneous cpm})} \times 100\%.
\]

where spontaneous release represents cpm in supernates from wells containing target cells and medium only, and maximum release represents cpm in supernates from wells containing target cells in medium with 5% Triton X100. Spontaneous release was always <20% of maximum release. The SD of triplicate wells was less than 10%.

**Human IFN-y Bioassay.** Supernatants were collected from MTLR after 5 days of culture and frozen at —20°C until assay. The bioassay for human IFN-y was based on the ability of the IFN-y to inhibit the cytopathic effect of the encephalomyocarditis virus on A549 cells (25).

**IL-2 Production and Bioassay.** PBLS were stimulated with melanoma cells (ratio of PBLS:tumor cells, 20:1) for different periods and the supernatants were collected at indicated times. IL-2 activity was determined by the ability to support the growth of CTLL cell line as described by Gillis et al. (26). CTLL was maintained in RPMI 1640 containing recombinant human IL-2 (5 units/ml). Prior to use for IL-2 bioassay, CTLL was washed twice and cultured for 24—30 h in medium without IL-2. CTLL (2 × 10^5 cells/well) was cultured with the supernatants containing IL-2 for 48 h. [3H]Thymidine was added (1 μCi/well) during the last 16—18 h of culture. IL-2 activity was calculated as described by Gillis et al. (26).

**Human GM-CSF Production and Bioassay.** PBLS were stimulated with B7^+ or B7^- melanoma cells as above, and the supernatants were collected after 4—5 days. GM-CSF was determined as described in the manual of the Human GM-CSF Immunoassay kit Quantikine purchased from R&D Systems, Inc. (Minneapolis, MN). Briefly, 200 μl of supernatants per well were added in duplicates to the 96-well plates coated with murine monoclonal antibody against human GM-CSF and incubated for 2 h at room temperature. After thorough washing, 200 μl of anti-GM-CSF antibody conjugated to horseradish peroxidase were added to each well and incubated for 2 h at room temperature. Substrate solution was added and incubated for 20 min, and the absorbance was determined using a microtiter plate reader set to 450 nm. GM-CSF concentrations were calculated by a computer-fitted GM-CSF standard curve.

**Statistical Analysis.** Determinations of statistical significance were done by ANOVA. P < 0.05 was considered statistically significant.

**RESULTS**

**Generation of Melanoma Cell Lines Expressing hB7.1 using a Retroviral Vector System.** The supernatants from G418-resistant PA317 clones were used to transfect melanoma cell lines DM443 and DM459. Following selection for 2 weeks in medium containing G418, the G418-resistant clones were isolated, and B7.1-positive cells were sorted by flow cytometry and cultured in MEM. These sorted transfectants, DM443/B7.1 and DM459/B7.1, have retained stable expression of B7.1 in culture for more than 1 year. The transfected tumor cell lines were stained with fluorescence-labeled antibodies against HLA class I, class II, ICAM-1 and B7.1. Following transfection, there were no changes in expression of HLA antigens or ICAM-1 (Fig. 1, A and B). Parental tumor cells expressed no measurable B7.1, whereas the transfected tumor cells expressed high levels of B7.1 costimulatory molecules at the cell surface (Fig. 1, A and B).

**B7.1 Gene-modified Melanoma Cell Lines Induce Potent Tumor-specific CTLS from Autologous PBLS in the Absence of Exogenous Cytokines.** PBLS from patient 443 or from patient 459 were stimulated in vitro for 5 days with irradiated (10,000 rads) nontransduced, control (empty vector) transduced, or transduced B7.1-expressing autologous tumor cells in the absence of exogenous cytokines to avoid nonspecific activation. The stimulated cells were then tested for cytoxicity against unmodified and B7.1-transduced autologous tumor cells. PBLS stimulated with parental tumor cells demonstrated no significant lysis of unmodified or B7.1-transduced autologous melanoma targets (Fig. 2, A and B). In contrast, PBLS stimulated with B7.1-modified tumor cells were lytic for B7.1-modified autologous targets and, most importantly, they also were highly lytic for unmodified autologous tumor cells (Fig. 2, A and B). PBLS stimulated with tumor cells transduced with empty vector demonstrated no lytic activity (data not shown). There was no significant expansion of T cells following stimulation with parental melanoma tumor cells (a mean recovery of 93% of the cultured PBLS). In contrast, recovery of T cells from cultures stimulated with B7-modified tumor cells averaged 166% of the cultured cells after 5 days.

Phenotype studies showed that the percentages of CD4^+ and CD8^+ T cells of autologous PBLS stimulated with DM443 and DM443/B7.1 were 49 and 30 (CD4 and CD8) and 46 and 43 (CD4 and CD8), respectively, and the percentages of DM459- and DM459/B7.1-stimulated autologous PBLS were 48 and 26 (CD4 and CD8) and 34 and 35 (CD4 and CD8), respectively. However, there is no difference in
CD3 percentage, which is 77–85%, between parental and B7.1-transfected tumor groups. These data indicate that B7.1-transduced tumor cells seem to preferentially induce CD8+ T cell proliferation.

**CTLs Generated by B7.1 Gene-modified Tumor Cells Are Tumor Specific and HLA Class I Restricted.** We and others have demonstrated that IL-2-dependent autologous melanoma tumor-specific CTLs recognize shared tumor-associated antigens in the context of self or shared HLA class I-A or -B region gene products (21, 27, 28). We, therefore, examined the restriction patterns of CTLs induced in the absence of exogenous IL-2 following stimulation with autologous, B7.1-modified melanomas. Their lytic patterns were tested on a panel of HLA class I-A-region matched or unmatched melanoma target cells. The results (Table 1) indicate that autologous stimulated DT443/B7.1 T cells (HLA-A2) are highly lytic not only for autologous tumor cells but also for HLA-A2-matched melanoma cells. Conversely, CTLs from B7.1 tumor-specific clone 13.5, which carries a different HLA-A gene (HLA-A1), were not superior to the autologous CTLs in killing HLA-A2-matched melanomas.
We have demonstrated in the past that in HLA-A2 melanoma patients, allogeneic HLA-A2-matched melanomas may, in the absence of autologous melanoma cells, be substituted as surrogate stimulator cells to induce IL-2 dependent, HLA-A2-restricted, melanoma-specific cytotoxic T cells (21, 27). We therefore tested the ability of B7.1-modified tumor cells to induce HLA-A2- or HLA-A29-restricted lysis in allogeneic PBLs from HLA-A2- or HLA-A29-expressing melanoma patients. PBLs from HLA-A2-expressing patient DM472 were stimulated with B7.1-modified, allogeneic HLA-A2 melanoma tumor cell line DM443/B7.1 in the absence of added IL-2. After 5 days, the cultured T cells were tested for lysis on a panel of HLA-A2-matched or unmatched melanomas. The T cells were lytic for three of three HLA-A2 melanomas tested, including DM472, the melanoma tumor cell line autologous to the stimulated PBLs, and demonstrated very low levels of activity against two of two non-A2 melanomas (Table 2). In addition, the T cells failed to lyse two of two HLA-A2-positive EBV cell lines and the K562 target cell. Similar results were obtained when HLA-A29-matched PBLs from DM259 were stimulated with HLA-A29-expressing, B7.1-modified, allogeneic DM459/B7.1 (Table 2). The T cells were lytic for both HLA-A29 targets tested and failed to lyse the HLA-A29 homozygous nonmelanoma EBV cell line SW5. We also tested the ability of B7-modified DM459 to elicit a cytotoxic, HLA-A29-restricted, melanoma-specific response by PBLs from a normal HLA-A29-expressing donor. The results (Table 2) indicate that, indeed, B7-modified DM459 but not parental DM459 can elicit an HLA-A29-restricted CTL response. These CTLs demonstrated a low to moderate level of cytotoxicity against the B-cell line YAR, which is homozygous for the HLA-A2 allele present on the B7-modified DM459 stimulator tumor cells. Although small compared to the HLA-A29-restricted response, this lysis may represent a low-level allogeneic response to the unshared HLA-A26 allele on the DM459 stimulator cells.

We also investigated further whether CTLs induced by B7.1 gene-modified tumor cells were HLA class I restricted. 51Cr-labeled targets were incubated with monoclonal antibody against HLA class I W6/32 for 30 min and then mixed with CTLs for cytolytic activity assay. The cytolytic activity of CTLs against HLA class I-matched targets was blocked significantly by anti-HLA class I antibody W6/32, but not by control antimurine class I (H-2Kb) monoclonal antibody (Fig. 3).

8 T. L. Darrow, S. Yang, and H. F. Seigler, HLA-A29 restricted recognition of shared melanoma TAA by autologous melanoma specific CTL, manuscript in preparation.

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**Fig. 2. Role of B7.1 in the generation and function of primary tumor-specific CTLs.**

A. PBL from a melanoma patient was stimulated with autologous tumor cells transfected with B7.1 gene, and the cytolytic activity was tested against 51Cr-labeled target cells DM443/B7.1 or DM459/B7.1 as described in “Materials and Methods.” B. PBL from a melanoma patient was stimulated with DM459/B7.1 for 5 days, and CTL activity was detected against 51Cr-labeled target cells DM459/B7.1 as described above. *P < 0.05 as compared with corresponding groups stimulated with parental melanoma cells.
CD8+ T Cells Are Primarily Responsible for Tumor Killing.
To investigate which subset of T cells was involved in the effector phase of tumor-specific cytotoxicity, the cultured cells generated by DM443/B7.1 from autologous PBLS were treated with complement (C''), C' + antibody OKT4 or C' + antibody OKT8 to deplete CD4 or CD8 T-cell subsets. The effectors were then tested for cytolytic activity against DM443. Depletion of CD8+ cells from the cultured cell populations led to significant reduction of lytic activity, indicating that CD8+ cells were primarily responsible for tumor killing (Fig. 5). Specific depletion of CD4 and CD8 cells was about 90% by flow cytometry (data not shown).

B7.1 Is Required for Generation of CTLs. CTLA4Ig, a chimeric protein composed of the extracellular domain of CTLA4 fused to human IgG Cy1, binds with high affinity to B7 and can block CD28-B7 interaction (32). To determine whether the transfected B7.1 contributes directly to the generation of tumor-specific CTLs in these primary cultures, we included CTLA4Ig in our in vitro CTL induction system. The CTLs were then tested on day 5 of culture. Autologous, melanoma-specific CTL induction elicited by both DM443/B7.1 (Fig. 6A) and DM459/B7.1 (Fig. 6B) were inhibited significantly in a dose-dependent manner by CTLA4Ig. Control antibody, human IgG, did not inhibit the generation of tumor-specific CTLs.

DISCUSSION
The findings of the present study are consistent with the results of Dohring et al. (19) and Sule-Suso et al. (20), which demonstrated that proliferative activation of allogeneic (17, 20) and autologous (20) lymphocytes and allogeneic CTLs (17, 20) could be induced by B7.1-modified tumors. Our study extends these findings to include an examination of the response to B7 gene-modified autologous melanomas and antigen specificity and HLA class I restriction of the CTLs. We show that: (a) CTLs are elicited readily from unfractionated autologous PBLS as well as autologous, purified CD8+ T cells following stimulation with autologous B7.1+ melanoma cells; (b) CTLs generated by B7.1-transfected melanoma cells from both autologous and HLA class I-matched allogeneic T cells are tumor specific and HLA class I restricted, efficiently lysing HLA class I-matched melanomas but not class I-matched EBV-transformed B cells or class I-unmatched melanoma cells; (c) CTLs elicited by HLA class I-matched, B7.1+ allogeneic tumor efficiently lyse not only the stimulating tumor cells but autologous tumor cells as well; and (d) B7.1 is required for CTL induction, but not required for tumor lysis. These results provide the additional evidence needed to support the use of HLA class I-matched, B7.1-transfected tumor cells as specific tumor vaccines for active immunization.

Previous in vitro work by us (21, 27) and by others (28) has demonstrated the need for low levels of exogenous IL-2 to support full T-cell activation and development of human melanoma-specific CTLs in vitro. Presumably, the added IL-2 provides the necessary second signaling to activate the T cells following engagement of their receptors with TAA in the context of HLA class I. Because it has been shown that costimulation by B7.1 may elicit the production of IL-2 by

### Table 1

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### Table 2

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### Notes
- PBLS were stimulated with HLA class I-matched allogeneic B7.1-modified melanomas for 5 days, and CTL were tested as described in the legend to Table 1.
- Percentage specific lysis.
- T cells generated by stimulating PBLS with DM443/B7.1 were named DT472-443/B7. Similar nomenclature was used for other T-cell lines. JM designates PBLS derived from a normal donor.
- P < 0.05 compared with corresponding non-HLA-A region-matched melanoma cells, and EBV-transformed B cells and K562 targets.
and then the effector cells were added to each well at an E:T ratio of 30:1 for 5 days. 5tCrlabeled target cells (5 × 10^6 cells/well) were incubated in antibody-treated groups. Incubation for another 4 h and C'I'L activity was determined as described in "Materials and Methods."*, P < 0.05 compared with corresponding medium groups and control antibody-treated groups.

In these experiments, there is no significant expansion of T cells and little cytotoxicity following stimulation with parental tumor cells. We have not tested the notion here that these conditions of T-cell tumor interaction in the absence of a second signal (B7 or exogenous IL-2) results in anergy of the cultured T cells. We have found in other studies not reported here that IL-2 can be added to T cells cultured with tumor cells as long as 3 days after culture initiation with no significant loss of subsequent T-cell antitumor activity. On the basis of the limited studies reported here, we cannot, however, exclude the possibility of in vitro tumor cell induced T-cell anergy.

B7.1-modified tumor cells are able to activate CD4+ T cells as demonstrated by Sule-Suso et al. (20), and activated CD4+ T cells may provide help to CD8+ T cells, presumably by providing IL-2 and other cytokines. It is also possible that CD8+ T cells can directly mount a cytolytic response if precursors of CD8+ CTLs encounter antigens and B7.1 on tumor cells in vivo. However, the possibility of involvement of CD4+ T-cell help (perhaps through production of IL-2) cannot be ruled out. Therefore, we examined the ability of purified CD8+ cells to generate CTLs in MLTR with B7.1-expressing tumors. Similar to the response elicited from unfractionated PBLs, B7.1-expressing tumor cells elicit a potent CTL response by purified CD8+ precursor PBLs. In these studies, the level of contaminating CD4+ T cells following depletion was very low (less than 1%). Thus,

<table>
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Fig. 3. Antibody to HLA class I blocks HLA-restricted lysis by CTLs stimulated with B7.1-modified melanoma. PBL472 (A2 and A3) was stimulated with DM443/B7.1 (A2) at a 20:1 ratio for 5 days. 5tCrlabeled target cells (5 × 10^6 cells/well) were incubated in the presence or absence of culture supernatants of anti-HLA class I hybridoma W6/32 or antimurine H-2Kb hybridoma (HB158, American Type Culture Collection) for 30 min. and then the effector cells were added to each well at an E:T ratio of 30:1. The cells were incubated for another 4 h and CTL activity was determined as described in "Materials and Methods."* P < 0.05 compared with corresponding medium groups and control antibody-treated groups.

Table 3  B7.1 costimulation of production of IL-2, IFN-γ and GM-CSF

<table>
<thead>
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<th>IFN-γ (units/ml)</th>
<th>GM-CSF (pg/ml)</th>
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<td>24 h</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>PBL259</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ DM459</td>
<td>0.12</td>
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</tr>
<tr>
<td>+ DM459B7</td>
<td>8.88</td>
<td>2.49</td>
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<td>PBL464</td>
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</tr>
<tr>
<td>+ DM443</td>
<td>1.59</td>
<td>0.79</td>
</tr>
<tr>
<td>+ DM443B7</td>
<td>4.13</td>
<td>0.95</td>
</tr>
</tbody>
</table>

* There is no IL-2 activity in PBL groups.

Supernatants were harvested after a 4-day culture. The data represent two independent experiments. IFN-γ activity in PBL groups is undetectable, and GM-CSF in PBL group is <10 ng/ml.

* P < 0.05 compared with corresponding parental tumor groups.
CD4+ T cells are not absolutely required in vitro in the presence of the B7 costimulatory molecule. Also in the current study, T-cell subset depletion using specific antibodies after the 5-day culture period showed that specific tumor killing following induction is mediated primarily by CD8+ T cells.

Because it has been reported that ICAM-1 on melanoma cells can act as melanoma antigen (35), we sought to test whether B7.1 expression on the surface of tumor cells can be recognized as a tumor antigen by CTLs generated by B7.1-expressing tumor cells. CTLs from both autologous PBLs and HLA class I-matched allogeneic PBLs stimulated with B7.1-expressing tumor cells were equally effective in lysing B7.1 modified tumor and parental tumor cells (Fig. 2). Furthermore, CTLs generated by B7.1-expressing tumor cells are cytolytic to B7.1-negative, HLA class I-matched melanoma cells, but not B7.1-positive, HLA class I-matched, EBV-transformed B cells (Table 2). In addition, in vitro administration of CTLA4Ig (32), an efficient inhibitor of B7 costimulatory interaction, effectively blocked the generation of specific CTLs in a dose-dependent manner (Fig. 6). These data indicate that B7.1 interaction plays an important role in induction of tumor-specific CTLs, but B7 binding is not necessary for tumor killing. Therefore, we conclude that the enhanced ability of B7.1-expressing tumors to elicit a CTL response is due to the ability of B7.1+ tumors to provide a costimulatory signal but not to provide a new antigen. This study also confirms previous data that the expression of B7.1 by the target cells is not required for the function of CTLs (17, 36).

The aim of transducing tumor cells with B7.1 is to modify their immunogenicity and increase their ability to fully activate tumor-specific T-cell precursors. We show here not only that B7.1-modified tumors are able to elicit CTLs from both autologous PBLs and purified CD8+ T cells, but that allogeneic, HLA-matched B7 gene-modified tumor cells can substitute as surrogate stimulator cells to elicit potent autologous, tumor-specific, HLA-restricted CTLs in vitro. We demonstrated previously, for patients whose autologous tumor cells are not available for use as immunogens or as in vitro stimulators for the generation of therapeutic CTLs, that allogeneic, HLA-matched melanomas, which express shared tumor antigens, may be substituted (21, 27). The work reported here is consistent with those previous findings, extends them to include HLA-A29 patients, and demonstrates the significant advantage of using autologous or allogeneic tumor cells transduced with the gene for B7.1.

In conclusion, these results indicate that modification of human melanoma tumor cells with the gene for B7.1 results in a dramatic increase in the ability of the tumor cells to induce in vitro tumor-specific, HLA-A region-restricted, cytotoxic T-cell responses from both autologous and HLA-A region-matched PBLs from melanoma patients. The data further suggest that such B7.1-modified tumor cells may be especially effective in vitro to induce lytic T cells for adoptive immunotherapy. The modified tumor cells may also be potent immunogens for use in a program of specific active immunotherapy for autologous or HLA-matched tumor patients.

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IN VITRO INDUCTION OF CTLs BY B7.1 MODIFIED MELANOMA


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Generation of Primary Tumor-specific Cytotoxic T Lymphocytes from Autologous and Human Lymphocyte Antigen Class I-matched Allogeneic Peripheral Blood Lymphocytes by B7 Gene-modified Melanoma Cells

Sixun Yang, Timothy L. Darrow and H. F. Seigler