Identification and Epitope Enhancement of a PAX-FKHR Fusion Protein Breakpoint Epitope in Alveolar Rhabdomyosarcoma Cells Created by a Tumorigenic Chromosomal Translocation Inducing CTL Capable of Lysing Human Tumors

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Abstract

Fusion proteins created by chromosomal translocations in tumors can create neoantigenic determinants at the breakpoint, which are unique to the tumor cells but shared by the vast majority of tumors of that histologic type. If the fusion protein is responsible for the malignant transformation, its expression cannot be lost by the tumor to escape immune responses against this tumor antigen. Here, we identify such a fusion protein breakpoint epitope in the PAX-FKHR fusion protein created by the t(2;13) translocation present in 80% of cases of alveolar rhabdomyosarcoma, a highly aggressive pediatric soft-tissue sarcoma. We use autologous dendritic cells pulsed with the RS10 breakpoint fusion peptide to raise a human CTL line from a normal healthy HLA-B7$^+$ blood donor specific for this peptide. These CTLs are CD8$^+$ (CD4$^-$CD56$^-$) and restricted by HLA-B7. These human peptide-specific CTL lyse human HLA-B7$^+$ rhabdomyosarcoma tumor cells. Therefore, the fusion protein is endogenously processed to produce this natural epitope presented by HLA-B7 and thus this peptide is a bone fide human tumor antigen. We also define a substitution that increases the affinity for HLA-B7 without loss of antigenicity. This epitope-enhanced peptide may serve as a candidate cancer vaccine for HLA-B7$^+$ patients with alveolar rhabdomyosarcoma. (Cancer Res 2006; 66(3): 1818-23)

Introduction

Most tumors express mutated or inappropriately expressed, nonmutated tumor-associated antigens (TAA) that often contain CTL epitopes. Yet, the immune system often remains incapable of overtaking the growth potential of the malignant cells. Currently, many approaches have been developed to obtain protective and therapeutic antitumor immunity. Active immunization strategies for treatment or prevention of tumors generally focus on the elicitation of TAA-specific CD8$^+$ CTL responses because these have the potential to generate durable and protective immunity including T-cell memory. The CTL epitopes are peptides usually 8 to 10 amino acids long with two to three primary anchor residues that interact with self MHC molecules whereas two to three alternative amino acid residues bind to the T-cell receptor (1). Yet, active immunizations in the form of peptides, proteins, DNA, either alone or with chemical adjuvants, thus far often fail to obtain the desired immune response (2). Alternatively, there is considerable interest in the use of dendritic cells for the delivery of TAAs. Dendritic cells have been recognized as very attractive adjuvants because they represent a specialized antigen-presenting cell population with the unique ability to activate naive CD4$^+$ and CD8$^+$ T cells and sustain primary immune responses.

Immune responses to most overexpressed nonmutated tumor antigens also may be diminished because of self-tolerance to the tumor antigen that is also expressed in some normal tissues or during ontogeny. Antigens with specific point mutations unique to the tumor avoid this pitfall but are often of limited utility because the mutations are different in each patient's tumor. Recently, we have been exploring another type of antigen unique to the tumor but shared among a majority of patients with that tumor type. These are the fusion proteins created by chromosomal translocations that play a role in oncogenesis of these tumors (3–5). The chromosomal translocation creates, at its junction or breakpoint, a fusion of amino acid sequences of two different proteins from the two parent chromosomes. Thus, this junction also may create neoantigenic determinants (i.e., epitopes that overlap this breakpoint and so exist only in the tumor fusion protein but not in either parent protein present in normal cells). In addition to being shared by most tumors of that type, these breakpoint epitopes also have the advantage that the fusion protein cannot be lost by the tumor to escape the immune response if the fusion protein is required for tumorigenesis, without loss of the malignant phenotype. We have previously shown proof of principle by defining a fusion protein breakpoint epitope created by a common chromosomal translocation in synovial sarcoma and by showing killing of tumor cells by specific CTL (5).

In the current study, we asked whether such a fusion protein epitope unique to the tumor could be found in a more common pediatric tumor, alveolar rhabdomyosarcoma, characterized by a t(2;13) translocation that creates the PAX-FKHR fusion protein believed responsible for tumorigenesis. This fusion gene merges the DNA binding domain of one transcription factor with the activation domain of a different one. This same breakpoint fusion protein is present in at least 80% of cases of pediatric or adult alveolar rhabdomyosarcoma (3, 4), making it a conserved potential tumor antigen target for immunotherapy of this malignancy. We used autologous dendritic cells from a normal healthy blood donor to raise a primary *in vitro* CTL response against a PAX-FKHR breakpoint peptide and derive a human CTL line that was able to kill human rhabdomyosarcoma cells. Furthermore, we identified a sequence alteration in the epitope that increased its binding to

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HLA-B7 and its antigenicity, which could serve as a more potent vaccine candidate to elicit such CTL in HLA-B7$^+$ patients with alveolar rhabdomyosarcoma.

Materials and Methods

Cell lines. C1R-B7, a specific transfectant of the B lymphoblastoid C1R cell line, which, in native form, expresses no endogenous HLA-A or HLA-B gene products (6), was a gift of William Biddison (National Institute of Neurological Disorders and Stroke, NIH, Bethesda, MD). T2-B7 is a specific transfectant of the hybrid B and T lymphoblastoid T2 cell line, which is deficient in *TAP1* and *TAP2* gene expression (7, 8), and which was a gift of Peter Cresswell (Yale University, New Haven, CT). The Rh5 alveolar rhabdomyosarcoma cell line was kindly provided by Dr. P. Houghton (St. Jude Children's Research Hospital, Memphis, TN). The RD and CTR embryonal rhabdomyosarcoma cell lines were obtained from the American Type Culture Collection and Dr. M. Tsokos (National Cancer Institute, Bethesda, MD), respectively. Cell lines were maintained in culture medium with heat-inactivated FCS (10% v/v). Culture medium consisted of RPMI 1640 (Cellgro, Bethesda, MD) containing L-glutamine (2 mmol/L), penicillin (100 IU/mL), streptomycin (100 μg/mL), nonessential amino acids (10 μL/mL), sodium-pyruvate (1.0 mmol/L), gentamicin (25 μg/mL), and 2-mercaptoethanol (50 μmol/L).

Peptides. Full-length peptides were purchased from Peptide Technologies Corp. (Gaithersburg, MD) and Multiple Peptide Systems (San Diego, CA) at >95% purity and were single peaks by reverse-phase high-performance liquid chromatography. Optimal epitopes were synthesized on an automated peptide synthesizer (Symphony Multiplex, Protein Technologies, Phoenix, AZ) using 9-fluoroenylmethyloxycarbonyl chemistry (9). The peptides were cleaved from the resin with trifluoroacetic acid. Purification to single peaks was achieved using reverse-phase high-performance liquid chromatography on bondapack reverse-phase C18 columns (Waters Associates, Milford, MA).

Flow cytometry. Conventional monoclonal antibody (mAb) staining was conducted in PBS containing 0.01% sodium azide on ice. Cells were labeled with FITC- or phycoerythrin-conjugated mAbs obtained from BD PharMingen (San Diego, CA). For each staining of interest, the appropriate isotype-matched control was included. All reagents were used at optimal concentration as determined experimentally. Flow cytometric analysis was done with a FACScan (BD Biosciences, Mountain View, CA). Data were collected on 5,000 to 10,000 viable cell events and analyzed with CellQuest software.

CTL assay. Specific cytotoxic activities were determined in a standard 4-hour ^{51}Cr release assay at various E/T ratios. Briefly, graded doses of viable effector cells were plated in triplicate in 96-well U-bottomed culture plates (Corning Glass, Corning, NY) and cocultured for 4 hours with sodium chromate–labeled (100 μCi; NEN, Boston, MA), peptide-pulsed (10 μmol/L) C1R-B7 target cells. In some experiments, the Rh5, RD, and CTR tumor cell lines were used as a target. Supernatants were collected, radioactivity measured, and specific lysis was calculated according to the following equation: percentage of specific cytotoxicity = (experimental cpm − spontaneous cpm) / (maximum cpm − spontaneous cpm) × 100.

Maximum ^{51}Cr release was determined from supernatants of lysed target cells incubated with Triton X-100 (5% v/v). Spontaneous release was determined from target cells incubated without added effector cells.

Peptide-HLA molecule binding assays. Peptide binding to HLA-B7 was assessed by previously described assays (10–13). The TAP1/TAP2–deficient T2 cell line (7, 8) transfected with the HLA-B7 gene was used. Cells were suspended in culture medium containing heat-inactivated FCS (2.5% v/v) and added to 96-well round-bottomed plates at 2×10^5 per well. Human β_2-microglobulin (Sigma Chemical Co., St. Louis, MO) was also added at 20 μg/well. Where appropriate, peptide was added to the desired concentration. The cells were then incubated overnight at 37°C in 5% CO_2, followed by washing with PBS containing NaN_3 (0.5% w/v and FCS 2% v/v). Next, cells were incubated on ice for 30 minutes in the presence of primary anti-HLA-B7 specific antibody (BB7.1 hybridoma culture supernatant; American Type Culture Collection, Manassas, VA), followed by washing and incubation for 30 minutes in goat anti-mouse immunoglobulin FITC (Becton Dickinson, Franklin Lakes, NJ). Analysis was done by flow cytometry.

Generation of human dendritic cells. Elutriated monocytes and lymphocytes were obtained from apheresed HLA-B7-positive subjects from the NIH normal adult donor pool. Monocytes were cultured for 7 days in 75-cm^2 culture flasks (Costar Corporation, Corning, NY) at 3.5×10^6/mL in culture medium containing heat-inactivated autologous plasma (10% v/v), human interleukin (IL)-4 (800 units/mL; R&D Systems, Minneapolis, MN), and human granulocyte macrophage colony-stimulating factor [GM-CSF; 50 ng/mL; Immunex Corp. (now Amgen, Seattle, WA)] at 37°C in an atmosphere of 5% CO_2 in air. At day 3, the cells were refed by removing 5 mL of the medium from the culture flasks and adding back 5 mL of fresh culture medium supplemented with cytokines (human IL-4, 400 units/mL; human GM-CSF, 25 ng/mL). At day 4, CD40 ligand trimer (Immunex) was added at 1 μg/mL (14). Cells were harvested on day 6 and stained for CD14, CD19, CD56, CD80, CD83, CD86, and HLA-DR antigens (see Fig. 1).

Generation of peptide-specific T-cell line. Lymphocytes from the pheresis of the normal adult HLA-B7$^+$ donor above were suspended in culture medium containing heat-inactivated autologous plasma (10%, v/v) and plated in a 24-well plate at 4×10^6 per well. Autologous dendritic cells were pulsed with RS10 peptide (10 μmol/L) in culture medium for 4 hours. Next, the dendritic cells were irradiated with 3,000 rad and added to the lymphocytes at 4×10^5 per well. The next day (day 1), cultures were supplemented with human IL-2 (12.5 units/mL), human IL-7 (2,400 units/mL), human IL-1β (150 units/mL), and human IL-12 (± 1 ng/mL). At day 7, the cells were harvested, washed, plated in a 24-well plate at 1.5×10^6 per well, and restimulated with irradiated RS10 pulsed autologous dendritic cells (1.5×10^5 per well). At day 8, the cells were supplemented with human IL-2 (12.5 units/mL) and human IL-7 (2,400 units/mL). Restimulations were done weekly using the same conditions. Cultures were checked every week for relative CD4, CD8, and CD56 expression and, if necessary, depleted of CD4$^+$ or CD56$^+$ cells by magnetic cell sorting using Magnetic Micro Beads (Miltenyi Biotec Midi-MACS, Auburn, CA).

Results

Identification of a peptide epitope and generation of human CTL. A chromosomal translocation-generated fusion protein breakpoint peptide, RS10 (SPQNSIRHNL; Table 1), was selected on the basis of predicted potential binding to HLA-B7 (1). It was found to bind HLA-B7 in a binding assay using stabilization of HLA-B7 molecules on the surface of T2-B7 cells that lack the TAP transporter and therefore express only short-lived empty HLA-B7 molecules, unless a peptide is present that can bind and stabilize them (see Fig. 6). To determine whether this peptide could elicit human CTL, we elutriated leukopheresed human mononuclear cells from an HLA-B7$^+$ normal healthy blood donor to separate a monocyte and a lymphocyte fraction. The monocyte fraction was converted into dendritic cells by growth in GM-CSF and IL-4 and maturation with CD40 ligand as described in Materials and Methods. These dendritic cells were found to be strongly positive for CD80 and CD86 costimulatory molecules, HLA-DR, and the maturation marker CD83 but were negative for CD14, CD19, and

Table 1. Peptides used in this study

Peptide	Amino acid sequence
RS10	SPQNSIRHNL
RS10-3A	SPANSIRHNL
RS10-5A	SPQNAIRHNL
RS10-6A	SPQNSARHNL
SS1	PQQRPYGYDQIMPKKPA

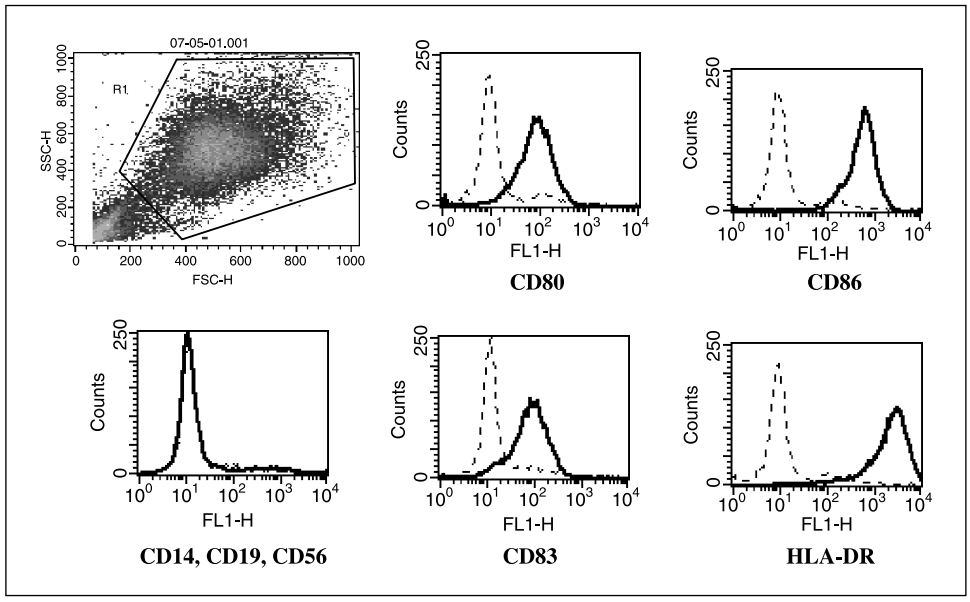

Figure 1. Flow cytometric phenotype analysis of the human dendritic cells used in this study. *Heavy line,* specific staining; *light line,* isotype control antibody staining.

CD56 (Fig. 1). These dendritic cells were then pulsed with the RS10 peptide and used to stimulate autologous lymphocytes from the same apheresis to generate a specific CTL line as described in Materials and Methods. The line was found to express CD8 but negative for CD4 and CD56 (Fig. 2).

The human CTL line was tested for ability to kill human C1R-B7 target cells pulsed with the specific RS10 peptide or a control SS1 peptide from synovial sarcoma that also binds HLA-B7 (ref. 5; Fig. 3). The lysis was clearly specific for the PAX-FKHR–derived RS10 peptide. Furthermore, the killing was restricted by the human HLA-B7 MHC class I molecule as shown by blockade of killing with antibody to HLA-B7 (Fig. 4).

Human CTL to the RS10 PAX-FKHR fusion peptide kill rhabdomyosarcoma cells expressing HLA-B7. To determine whether this RS10 epitope was naturally processed and presented by HLA-B7 in human tumor cells that express endogenous PAX-FKHR fusion protein, we tested the lytic ability of the RS10-specific CTL line against a rhabdomyosarcoma tumor cell expressing HLA-B7 (Rh5) and two control lines not expressing HLA-B7 (RD and CTR; Fig. 5). Because there are no known alveolar rhabdomyosarcoma lines that lack the specific translocation, the control for a target cell expressing HLA-B7, but lacking the specific epitope, is C1R-B7 pulsed with the control SS1 peptide shown in Fig. 3. Lack of killing of the translocation-positive but HLA-B7-negative targets confirms the HLA-B7 restriction of the CTL line. Clear specific lysis of the Rh5 cells compared with the other control tumor cells indicated that the RS10 epitope was indeed naturally endogenously processed and presented in unmanipulated human tumor cells and that CTL raised against this epitope could kill human tumor cells.

Epitope enhancement of the RS10 epitope. To maximize immunogenicity, it is often helpful to modify the sequence of an epitope to increase the affinity for the relevant MHC molecule,

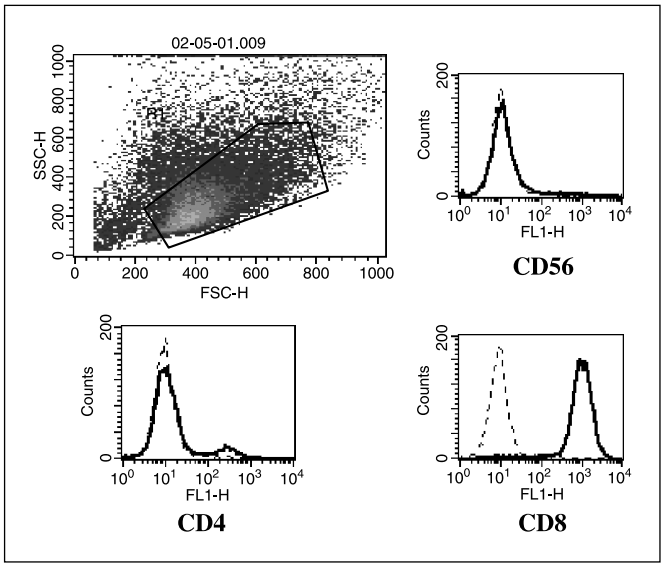

Figure 2. Flow cytometric phenotype analysis of the human CTL line used in this study. *Heavy line,* specific staining; *light line,* isotype control antibody staining.

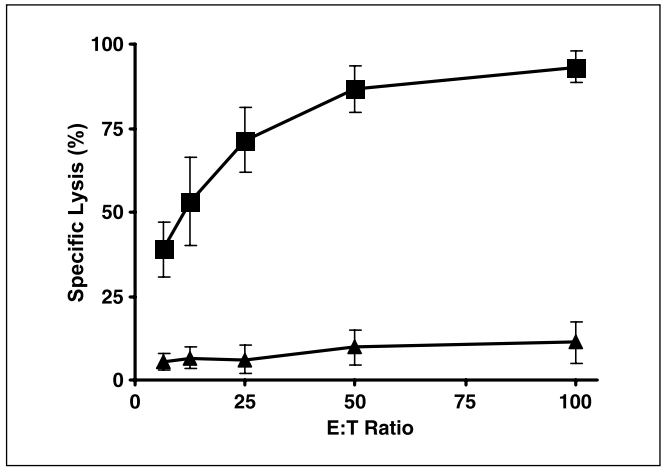

Figure 3. Lysis of RS10-pulsed C1R-B7 targets by RS10-specific CTLs. The control targets were C1R-B7 targets pulsed with an irrelevant SS1 peptide that also binds HLA-B7. This experiment was done five times with similar results. ■, C1R-B7 + RS10; ▲, C1R-B7 + SS1.

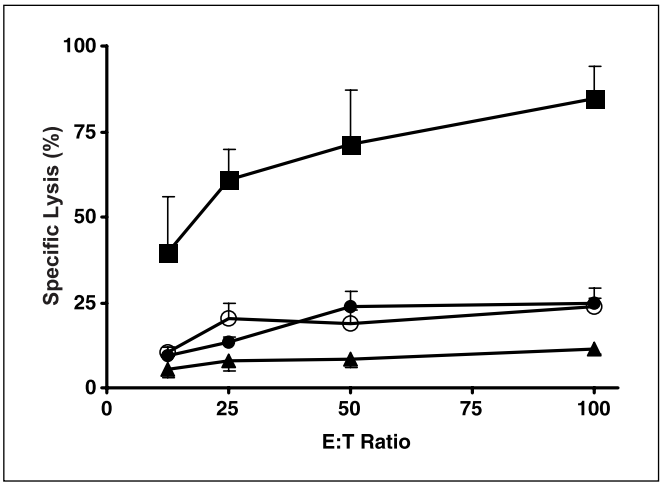

Figure 4. Blocking of lysis of C1R-B7 targets pulsed with RS10 peptide by anti-HLA-B7 antibody. The SS1 peptide was used as a control as in Fig. 3. The experiment was repeated twice with comparable results. ■, C1R-B7 + RS10; ▲, C1R-B7 + SS1; ●, C1R-B7 + RS10 + anti-HLA-B7 (20% v/v); ○, C1R-B7 + RS10 + anti-HLA-B7 (10% v/v).

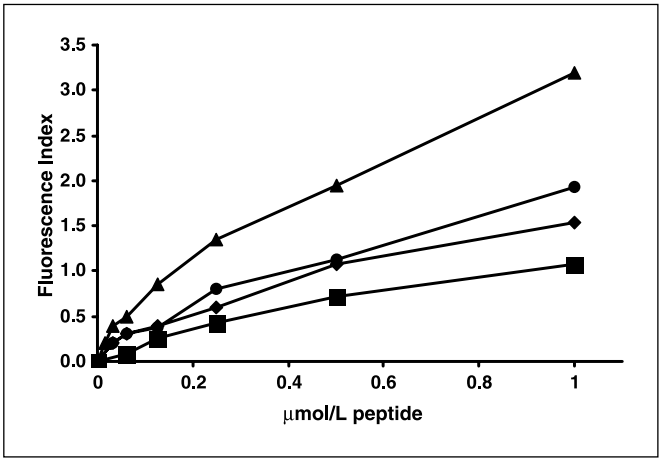

Figure 6. Binding of rhabdomyosarcoma-derived peptides to HLA-B7 molecules, measured using T2-B7 cells (n = 3). Fluorescence index is the ratio of HLA-B7 expression on TAP-deficient T2-B7 cells in the presence versus the absence of the peptide, minus 1. ■, RS10; ▲, RS10-3A; ●, RS10-5A; ♦, RS10-6A.

in a process we call epitope enhancement (15–23). Such improvements in binding affinity can sometimes be achieved by replacement of an amino acid residue causing an adverse interaction with one that has a small, neutral side chain, such as alanine (15–17, 19, 24). To screen for such a possibility, a series of peptides with Ala substitutions at each position in RS10 was synthesized and tested for binding to HLA-B7 in a T2-B7 binding assay. Only the variant with the Ala substitution at position 3 (RS10-3A) showed a substantially higher binding affinity for HLA-B7 than the natural RS10 peptide, although the peptides with Ala substitutions at positions 5 and 6 (RS10-5A and RS10-6A) showed binding with slightly higher affinity than the wild-type peptide (Fig. 6). The concentration required for a 50% increase in HLA-B7 expression was decreased from ~0.40 μmol/L for RS10 to ~0.08 μmol/L for the RS10-3A variant whereas RS10-5A and RS10-6A were intermediate, requiring ~0.2 μmol/L peptide to increase expression by 50%.

To be sure that the epitope-enhanced peptide, with increased binding affinity for the HLA molecule, had not simultaneously lost recognition by the T cell, we examined the ability of the RS10-specific human CTL to kill C1R-B7 targets pulsed with the RS10-3A peptide (Fig. 7). The specific killing observed confirmed that the enhanced epitope was not so altered in the surface presented to the T-cell receptor that it had lost recognition by the human CTL specific for the wild-type RS10 sequence. This enhanced peptide may therefore serve as a candidate vaccine to elicit specific CTL immunity in HLA-B7$^+$ patients with alveolar rhabdomyosarcoma without the concern that self-tolerance could dampen the response or that the tumor could escape by losing the fusion protein.

Discussion

In this study, we found that the PAX-FKHR fusion protein, created by the t(2;13) chromosomal translocation present in the vast majority of alveolar rhabdomyosarcoma tumors, contains a neoantigenic determinant at the breakpoint junction that is

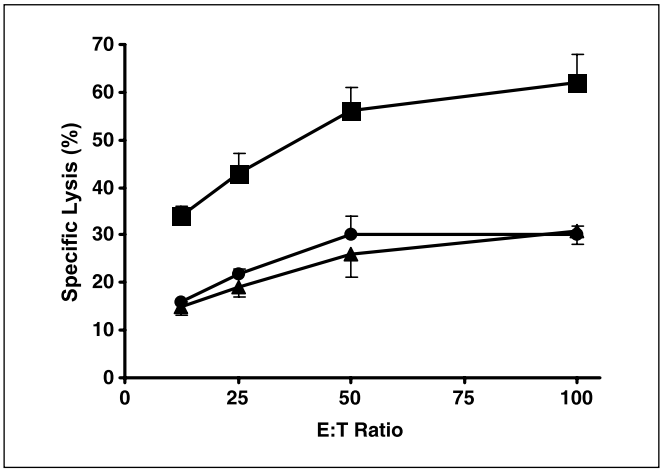

Figure 5. CTLs are able to lyse rhabdomyosarcoma cells expressing HLA-B7 (Rh5). The control targets were rhabdomyosarcoma cells not expressing HLA-B7 (RD and CTR). The experiment was repeated twice with comparable results. ■, Rh5; ▲, RD; ●, CTR.

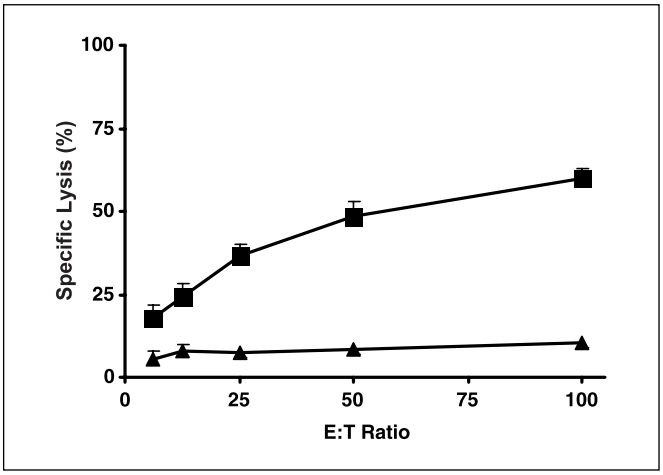

Figure 7. RS10-specific T-cell line cross-reacts with RS10-3A. The SS1 peptide was used as a negative control as in Fig. 3. The experiment was repeated twice with comparable results. ■, C1R-B7 + RS10-3A; ▲, C1R-B7 + SS1.

presented by HLA-B7 to human $CD8^+$ T cells. These $CD8^+$ T cells can kill human tumor cells expressing the fusion protein, indicating that this epitope is endogenously processed and presented and that this is therefore a bone fide tumor antigen that may be an Achilles heel for the rhabdomyosarcoma cells, allowing human CTL to pick out and destroy them from amidst normal cells that lack the translocation. This epitope also has the advantage that it is critical for the malignant transformation, and thus it cannot be lost by a tumor cell to escape the immune response without loss of the malignant phenotype. We have also identified a sequence modification in the epitope that results in an increase in affinity for the HLA-B7 molecule without loss of recognition by CTL specific for the wild-type epitope. This epitope-enhanced peptide may be a more potent vaccine to protect HLA-B7-positive individuals against alveolar rhabdomyosarcoma.

It is of interest that a recent study addressing the same issue did not find any such neoantigenic determinants in the breakpoint region of the PAX-FKHR fusion protein presented by HLA-A1, HLA-A2, or HLA-A3 molecules (25). However, this study did not examine HLA-B7 binding. The current investigation revealed the existence of such a neoantigenic determinant presented by HLA-B7; however, in concert, these studies indicate that only a minority of the population will have the correct HLA molecule to present this peptide. HLA-B7 is present in nearly a quarter of the population. Based on these findings, two possible scenarios may be considered. First, it is possible that individuals expressing HLA-B7 will have natural T-cell immunosurveillance against tumors expressing this neoantigenic determinant in the PAX-FKHR fusion protein, and thus will be more resistant to the development of alveolar rhabdomyosarcoma, or will be among the rare individuals with alveolar rhabdomyosarcoma who lack this fusion protein. Our results provide the basis to propose an epidemiologic study to determine whether alveolar rhabdomyosarcoma with the predominant t(2;13) translocation is less common in $HLA\text{-}B7^+$ individuals than in individuals lacking this class I HLA molecule. Second, it is possible that natural immunosurveillance against this epitope is inadequate to suppress the development of such tumors, perhaps because the tumor itself is not very immunogenic, and that these will be found in $HLA\text{-}B7^+$ individuals with a frequency similar to that in the whole population. In that case, it is possible that a vaccine containing this peptide might elicit a more effective CTL response that could cause tumor regression in patients who are positive for HLA-B7. Vaccines can be designed to induce qualitatively as well as quantitatively more effective CTL responses by incorporating cytokines and costimulatory molecules to induce higher-avidity CTLs (26–28), which are more effective at eliminating viral infections (29–31) and tumors (32, 33). A push-pull approach (34), in which such cytokines and costimulatory molecules are used to push the response and blockade of negative regulatory mechanisms is used to pull the response (23, 35, 36), can be applied to increase tumor immunity raised by a cancer vaccine (37). We propose to examine the frequency of HLA-B7 in alveolar rhabdomyosarcoma patients (mostly pediatric and young adult patients) and, if the second scenario holds true, to carry out a trial of such an engineered vaccine to treat such patients.

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